Distribution and Nucleocytoplasmic Transport of the Thyroid Hormone Receptor and Variants

A thesis submitted in partial fulfilment

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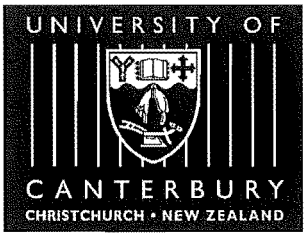

Caroline F. Bunn

1999

In memory of my mother,

Robyn Louise Bunn.

You remain my inspiration.

"To understand the utterances that emanate from the world's secret internal sanctuaries takes a lifetime of dedication, study and patience. There are those who would say that it takes more than one lifetime to acquire such skills. After all, anyone can hear the Raven calling on a grey day in the fields, but how many will ever learn the skill of listening to what the bird has said?"

Caíseal Mór

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Abstract

The thyroid hormone receptor (TR) is a member of the nuclear hormone receptor (NHR) superfamily. Members of this superfamily exhibit diverse subcellular distributions and nucleocytoplasmic transport mechanisms. TR exhibits a dual role as a repressor (or activator) in the absence of thyroid hormone (T_3), and an activator (or repressor) of gene transcription in the presence of T_3, thus implying constitutive nuclear localisation and persistent contact of TR with DNA. To investigate the complexity of the cellular response to T_3, the nucleocytoplasmic distribution of TR, its viral oncogenic variant (v-ErbA), and a DNA-binding mutant (C122A) were studied, using SDS-PAGE and fluorography to detect radiolabelled proteins extracted from microinjected *Xenopus* oocytes, and green fluorescent protein (GFP) fusions to study trafficking in transfected NIH/3T3 cells. Surprisingly, after cytoplasmic injection only 39-40% of TRα, TRβ, and C122A was localised to the oocyte nucleus, increasing to 45-51% in response to T_3. After nuclear injection, 62% of TRα was retained in the nucleus in the absence of T_3 and 85% in the presence, suggesting that more intranuclear binding sites are available for interaction with T_3-bound receptor. Only 10% of v-ErbA was localised to oocyte nuclei after cytoplasmic injection, due to association of v-ErbA with hsp90. A similar cytoplasmic subpopulation of v-ErbA/hsp90 in mammalian cells has been previously reported. The distribution of v-ErbA was unaffected by T_3. Nuclear accumulation in oocytes is not due to DNA binding alone, since C122A distribution was similar to TR. Interestingly, import of TR and C122A, but not v-ErbA, in oocytes was energy and temperature independent and not inhibited by wheat germ agglutinin. However, export of TRα, TRβ, and C122A was temperature dependent. In contrast to *Xenopus* oocytes, EGFP-tagged TRα localised entirely to the nuclei in transfected mouse cells, even in energy-depleted and chilled cells, and EGFP-tagged C122A remained extranuclear. Both TRα and EGFP-tagged TRα were shown to be associated with the nuclear matrix in biochemically fractionated NIH 3T3 cells. C122A and EGFP-tagged C122A were not found in the nuclear fractions, confirming the observations in living cells. A universal model for TR localisation is proposed to account for the observations made in the two cell systems. Since nuclear localisation of TR, v-ErbA, and C122A is not constitutive in *Xenopus* oocytes, this provides a model system for reconstituting regulatory networks affecting nuclear or cytoplasmic retention of TR and its variants *in vivo*.

Abbreviations

a.a.	Amino Acid
Ab	Antibody
AEV	Avian Erythroblastosis Virus
AR	Androgen Receptor
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
DBD	DNA Binding Domain
DNA	Deoxyribonucleic Acid
D-PBS	Dulbecco's Modified Phosphate Buffered Saline
EGFP	Enhanced Green Fluorescent Protein
ER	Oestrogen Receptor
GFP	Green Fluorescent Protein
GlcNAc	*N*-Acetylglucosamine
GR	Glucocorticoid Receptor
GTP	Guanosine Triphosphate
HBD	Hormone Binding Domain
HIV-1	Human Immunodeficiency Virus Type-1
hnRNP	Heterogeneous nuclear RNP
HRE	Hormone Responsive Element
hsp	Heat Shock Protein
kDa	Kilo Daltons
MR	Mineralocorticoid Receptor
mRNA	Messenger Ribonucleic Acid
NES	Nuclear Export Signal
NHR	Nuclear Hormone Receptor
NLS	Nuclear Localisation Signal
NM (-IF)	Nuclear Matrix (- Intermediate Filaments)
NPC	Nuclear Pore Complex
NuMA	Nuclear Mitotic Apparatus Protein
PBS	Phosphate Buffered Saline
PR	Progesterone Receptor
RAR	Retinoic Acid Receptor
RNP	Ribonucleoprotein
rRNA	Ribosomal Ribonucleic Acid
RXR	Retinoid X Receptor
snRNA/RNP	Small Nuclear Ribonucleic Acid/Ribonucleoprotein Particle
SV40	Simian Virus 40
T_3	3,3',5-triiodo-L-thyronine
TFIIIA	Transcription Factor IIIA
TR	Thyroid Hormone Receptor
TRE	Thyroid Hormone Responsive Element
VDR	Vitamin D Receptor
WGA	Wheat Germ Agglutinin

Chapter 1

General Introduction

1.1 The nuclear hormone receptor superfamily

The recognition of an increasing number of genetic diseases has focused the attention of researchers on the disruption of cell growth and development. However, it is difficult to envisage curative agents being developed without a substantial increase in the understanding of how cells function normally. Nuclear hormone receptors (NHRs) are involved in many aspects of cellular development at the level of gene expression. They act as highly abbreviated signal transduction pathways and upon binding specific sites in the genome, regulate transcription of adjacent genes (Evans, 1988).

The members of this superfamily, including the thyroid hormone receptor (TR), display a diversity of distributions within the cell and variation in transport mechanisms between the cytoplasmic and nuclear compartments. The exact cellular distributions of these receptors, in the absence or presence of hormone, are still an issue due to variations in results obtained with a variety of techniques in different cell types. Recently it has been recognised that the transport of receptors does not appear to be a static, unidirectional process, but also seems to reflect shuttling of receptor molecules between the nucleus and cytoplasm. The refinement and development of techniques to determine receptor distribution and transport has lead to a reassessment of previously accepted studies.

This thesis focuses on the distribution and transport of TR between the cytoplasmic and nuclear compartments of two model systems; *Xenopus* oocytes and cultured NIH 3T3 cells. Distribution and transport of two variants of TR are characterised: the viral oncogenic protein (v-ErbA), and a DNA-binding mutant (C122A).

1.1.1 An introduction to members of the superfamily

In addition to TR, the members of the NHR superfamily include receptors for progesterones, oestrogens, androgens, glucocorticoids, mineralocorticoids, vitamin D, and retinoids (reviewed in Tsai and O'Malley, 1994). The receptors that comprise this superfamily are transcription factors that bind to specific cis-acting DNA sequence elements in the genome or hormone responsive elements (HREs). Cis-acting sequence elements are found adjacent to the genes they are involved in regulating. Subsequent to HRE binding, NHRs regulate transcription of these adjacent target genes in response to ligand binding (Evans, 1988). For example, TR binds thyroid responsive elements (TREs). It activates or represses gene transcription in both the absence and presence of thyroid hormone. The products of these target genes form a network of proteins involved in control of cell growth and differentiation, and therefore morphogenesis of the animal.

Nuclear hormone receptors contain several domains that are differentially conserved between members. Figure 1.1 displays the domains of a generalised member of the NHR superfamily and highlights the specific regions involved in nuclear transport, receptor dimerisation, and heat shock protein 90 (hsp90) binding. Domains A and B are involved in modulating the *trans*-activation function of the receptor; domain C contains the DNA binding domain (DBD) which includes two zinc finger motifs (Figure 1.2); domain D is a 'hinge' region; whilst domain E is required for hormone binding (HBD), dimerisation of receptor molecules, and regulation of transcription (Laudet *et al.*, 1992; Nelson *et al.*, 1993).

The N-terminal regions of NHRs are variable amongst members of the superfamily. A portion of this domain, together with the DBD and part of the HBD, is responsible for *trans*-activation (Guiguere *et al.*, 1986; Kumar *et al.*, 1987; Godowski *et al.*, 1988; Webster *et al.*, 1988). Nuclear localisation and hormone binding may also be involved in *trans*-activation functions providing further means of regulating NHR activity (Kumar *et al.*, 1987; Picard and Yamamoto, 1987). The short, cysteine-rich central region is well conserved and the C-terminal half is relatively well conserved. The cysteine residues in the central region of the receptor form two zinc fingers, each with a zinc atom tetrahedrally co-ordinated to four of the

cysteine residues (Miller *et al.,* 1985). Separately the zinc fingers are unable to associate with DNA, but together they are responsible for DNA binding (Evans and Hollenberg, 1988; Freedman *et al.,* 1988). Overlapping this region and extended into the C-terminal region are the generalised nuclear localisation signals (NLSs) for receptor import, shown in Figure 1.1. These sequences are elaborated on in Section 1.2.4. The short region responsible for association with hsp90, indicated below, is discussed in Section 2.1.4. TR functions as dimers in association with TREs (Section 1.1.2); that is, the receptor dimerises with another molecule of itself (homodimer) or another NHR (heterodimer). The dimerisation domain is shown in Figure 1.1.

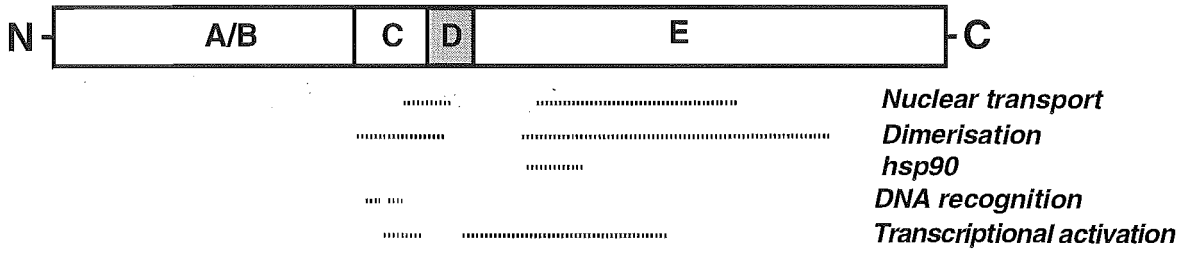


Figure 1.1 - Schematic of a generalised nuclear hormone receptor. ***Domains are designated by letters A to E. A and B are variable regions involved in trans-activation of the receptor, C contains the DBD, D is a hinge region, and E contains the HBD, dimerisation, and transcriptional regulation regions (as described in Section 1.1.1). The amino and carboxyl termini are represented by N and C, respectively. Hashed lines indicate the regions associated with the displayed receptor functions.***

1.1.2 The thyroid hormone receptor[1]

The TR gene was initially identified due to its homology to a viral oncogene, v-*erbA* (Weinberger *et al.,* 1986). The similarity of v-ErbA protein to another NHR, the glucocorticoid receptor (GR), led to this investigation. A second group of researchers simultaneously reported finding two genes encoding the cellular thyroid hormone receptor (Sap *et al.,* 1986). These two TR genes (or c-*erbA* genes) were isolated from chicken embryo and human placental tissue, respectively. Several isoforms of the c-*erbA* gene have been

[1] c-*erbA* and v-*erbA* refer specifically to the genes encoding the proteins c-ErbA and v-ErbA, respectively. Additionally, c-ErbA is referred to as TR.

identified in a variety of animals, including *Xenopus laevis* (Damm *et al.,* 1989; Yaoita *et al.,* 1990). The isoforms range in size from 45 to 55 kDa, including the two most common c-ErbA isoforms, designated TRα and TRβ. The TRβ gene can be differentially spliced to produce a number of proteins. Analysis of the two distinct isoforms isolated by Yaoita and colleagues identified 97% homology shared by the α-isoforms, whilst the β-isoforms were seen to be produced by alternative splicing of transcripts, transcribed from up to eight exons (Yaoita *et al.,* 1990). A comparison between the two most common receptor isoforms (TRα and TRβ) is displayed in Figure 1.3.

Two thyroid hormones, 3,5,3'-triiodo-L-thyronine (T_3) and L-thyroxine (T_4), are critical for development, differentiation, and morphogenesis in animals (Graupner *et al.,* 1989, Ribeiro *et al.,* 1995), although TR only associates with T_3. Molecules of TR bind as homodimers or heterodimers formed with NHR partners, most commonly retinoid X receptor (RXR) but also retinoic acid receptor (RAR), to TREs in target genes. In contrast to other members of the NHR superfamily, a cytoplasmic population of TR has not been demonstrated, although some TR-related proteins have been shown to have an extranuclear localisation (discussed in Section 2.1.2). Therefore, all of the classical proposed modes of action were based on constitutive contact of TR with TREs. Indeed, it has been shown that in the absence of hormone, TR can bind to its response elements and repress or activate transcription (Damm *et al.,* 1989; Graupner *et al.,* 1989).

Four *trans*-activation regions have been identified in TR that control transcriptional activation of genes containing a TRE. A constitutive *trans*-activation domain is situated in the N-terminal region (Hadzic *et al.,* 1995; Hollenberg *et al.,* 1995), whilst three hormone inducible domains are located in the C-terminal region (Baniahmad *et al.,* 1995). In mammalian cells, TR acts as an activator in the presence of hormone through the hormone inducible C-terminal domains. In addition, TR acts a repressor of gene transcription in the absence of hormone (Damm *et al.,* 1989; Sap *et al.,* 1989; Baniahmad *et al.,* 1990; 1992; Zenke *et al.,* 1990). Hormone independent activation has also been observed (Forman *et al.,* 1988; Forman and Samuels, 1990; Privalsky *et al.,* 1990; Saatcioglu *et al.,* 1993). The repressor action of TR has been isolated to the hinge region of the receptor and is distinct from the activation domains (Damm and Evans, 1993). At a further level of regulation,

release of a TR-bound corepressor (N-CoR), after ligand binding, and subsequent binding of co-activators are responsible for T_3-induced *trans*-activation (reviewed in Shibata *et al.*, 1997). A second co-repressor (SMRT: silencing mediator for RAR and TR) associates with unliganded receptor in solution, as well as unliganded receptor bound to DNA (Chen and Evans, 1995; Chen *et al.*, 1996; Sande and Privalsky, 1996), which is also the case for N-CoR.

1.1.3 v-ErbA oncoprotein

The v-*erbA* gene is one of two oncogenes carried by the avian erythroblastosis virus (AEV) that induces erythroleukemias in chickens (Graf and Beug, 1983). It is found on a 3 kb cell-derived insert, flanked by remnants of the retroviral structural genes, *gag* and *env* (Graf and Beug, 1983). The two proteins encoded by the cell-derived genes of AEV, v-ErbA and v-ErbB, appear to act cooperatively to induce transformation of cultured chicken erythroblasts and to cause acute erthryoleukemia in chickens (Beug *et al.*, 1982; Sealy *et al.*, 1983). The v-*erbB* gene is a truncated form of chicken epidermal growth factor and v-ErbA is derived from the cellular c-*erbA* gene. The v-ErbA protein is not required for oncogenic transformation of cells. It does, however, enhance v-ErbB action by modifying growth in the infected fibroblasts and by blocking differentiation of infected erythroid cells, so they remain in an immature and highly proliferative state (Graf and Beug, 1983; Gandrillon *et al.*, 1989). Deregulation of the control of cell division leads to the development of leukaemia, which is a form of cancer of progenitor cells that produce the circulating cells in the blood and lymphatic system (reviewed in Watson *et al.*, 1987).

The v-*erbA* gene produces a 75 kDa *gag*/v-*erbA* fusion protein that binds to TREs (Sap *et al.*, 1986; Damm *et al.*, 1989). However, v-ErbA, unlike its cellular homologue, is unable to bind thyroid hormone at physiological concentrations. The inability of the receptor to bind hormone is due to multiple mutations in the carboxyl-terminal domain (Sap *et al.*, 1986; Munoz *et al.*, 1988). Twelve amino-terminal amino acids of TR are replaced by the *gag* gene product in v-ErbA (Damm *et al.*, 1989). The structure of v-ErbA is compared with TR in Figure 1.3.

As v-ErbA retains its DNA binding activity, it is able to prevent TR from binding to TRE sequences in the DNA. At high levels, therefore, v-ErbA can repress the activation of specific genes involved in cell differentiation. Examples of genes repressed by the over expression of v-ErbA include CAII, encoding carbonic anhydrase II, the erythrocyte anion transporter gene in chickens (Schroeder *et al.,* 1990; Disela *et al.,* 1991), and the interstitial collagenase gene found in chicken embryo fibroblast cells (Desbois *et al.,* 1991). Due to structural changes, relative to TR, v-ErbA protein appears to act as a dominant negative repressor that interferes with the actions of its cellular homologue during transformation of normal cells to cancerous cells (Damm *et al.,* 1989).

However, this action of v-ErbA depends on the cellular context. In *Xenopus* oocytes, v-ErbA has been reported to act as a constitutive activator, and does not affect TR regulation of a tripartite rat growth hormone gene TRE (Nagl *et al.,* 1995). In the presence of v-ErbA, retinoic acid and thyroid hormone do not induce the expected induction of apoptosis in erythrocytic progenitor cells (Gandrillon *et al.,* 1994). Activated RXR is repressed by both TRα and v-ErbA, although the repression is distinct and dependent on both cell type and the HRE to which RXR binds (Wahlström and Vennström, 1998). Only TR represses efficiently in HeLa cells, whilst both TRα and v-ErbA were equally effective in JEG cells. Interestingly, in *Xenopus* oocytes, ultrastructural changes induced by v-ErbA were not mimicked by an *in vitro* generated dominant negative mutant of TR (Nagl *et al.,* 1997). Suppression of another steroid hormone receptor, RAR, may also be an important element in the mechanism of v-ErbA action (Chen and Privalsky, 1993).

1.1.4 A DNA-binding mutant of TR

As introduced in Section 1.1.1, the members of the NHR superfamily contain a highly conserved DNA binding domain. Within this domain are two zinc finger motifs (as displayed in Figure 1.2), and immediately C-terminal to each finger is an α-helix, which is involved in folding of the zinc fingers into a compact globular structure (Hard *et al.,* 1990a; 1990b; Schwabe *et al.,* 1990; Remerowski *et al.,* 1991). Each α-helix has a specific function. The

helix adjacent to the first finger is responsible for sequence specific DNA recognition, whilst the second contacts the DNA backbone (Danielsen *et al.*, 1989).

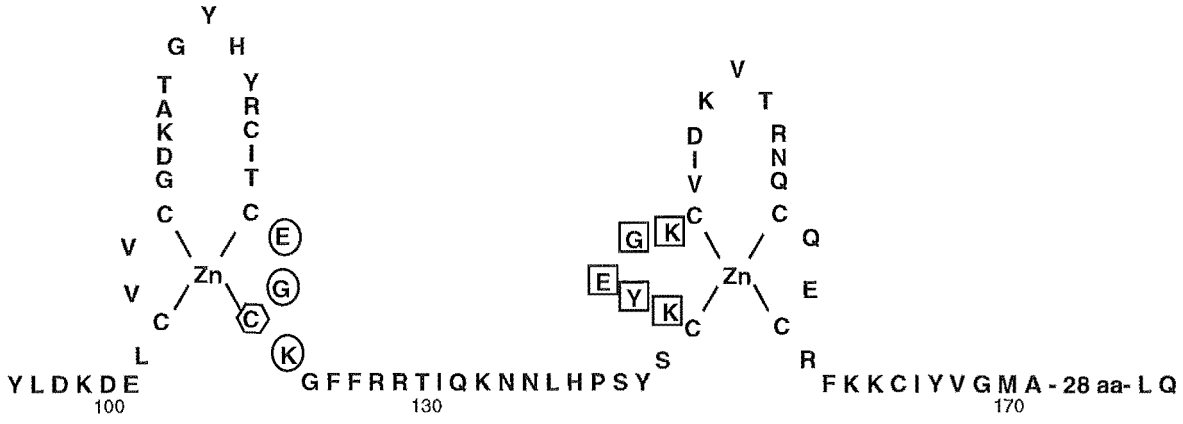


Figure 1.2 - Zinc finger domain of TR. ***The zinc finger motif present in the DBD is displayed. The hexagonally outlined cysteine is replaced by an alanine in TRβ DNA-binding mutant C122A.***

At amino acid position 122 of TRβ, a cysteine residue is one of the four involved in the tetrahedral co-ordination of a zinc atom (as shown in Figure 1.2). An *in vitro*-generated cysteine to alanine transition at this position in TRβ produces a receptor defective in DNA binding, simply designated C122A (Nelson *et al.*, 1993). A comparison of the four TR variants used in the investigations described in this thesis, including TRβ and C122A, is displayed in Figure 1.3. As C122A represses TR-mediated gene transcription, it has been suggested that the DNA-binding mutant acts by forming inactive heterodimers with TR, although this has not been demonstrated (Nelson *et al.*, 1993). In addition, the subcellular distribution of C122A was not analysed. Therefore, C122A provides an essential tool for investigating whether localisation of TR is due entirely to its ability to bind DNA, or whether there are other sites available for receptor interactions within the nucleus of a cell.

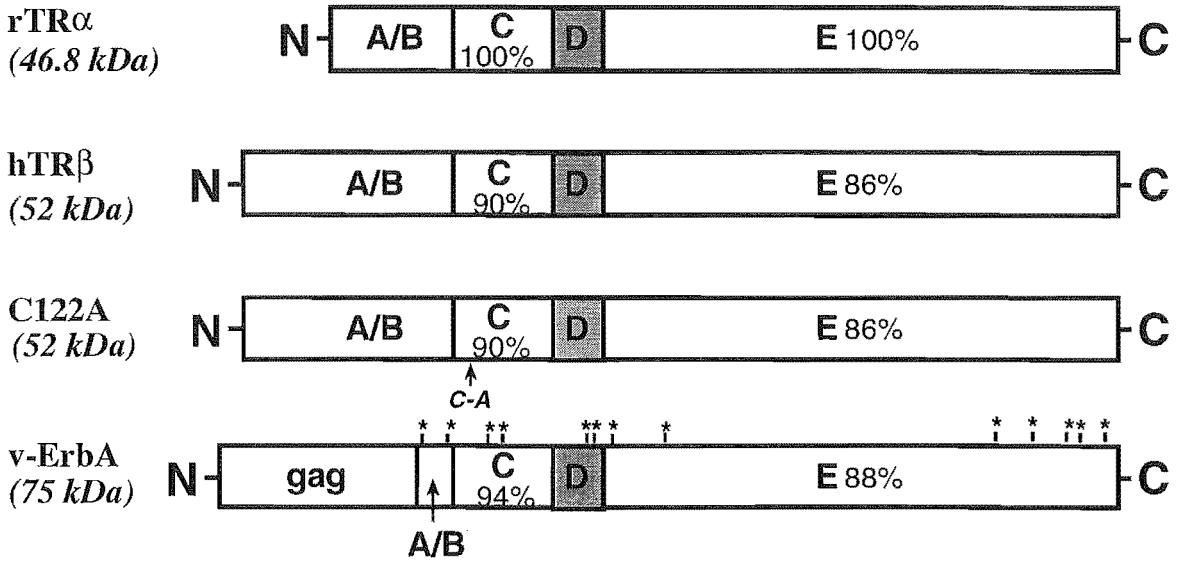


Figure 1.3 - Modular structures of the receptors TRα, TRβ, C122A, and v-ErbA. ***Receptor domains are designated as in Figure 1.1. Percentage homology of receptor domains C and E are shown with respect to rTRα. The transition at position 122 from C (in TRβ) to A (in C122A) is shown; details are presented in Figure 1.2. Asterisks show the position of mutations in v-ErbA in comparison to rTRα. (rTRα: rat TRα; hTRβ: human TRβ). Protein sequences are compared in Appendix I.***

1.2 Nucleocytoplasmic transport

1.2.1 The basis of nucleocytoplasmic transport

Eucaryotic cells can be thought of as divided into two compartments, dependent upon function. The nuclear membrane forms a selective barrier between the genetic material of the nucleus and the site of protein synthesis in the cytoplasm. The movement of macromolecules and complexes between these two compartments is a regulated process that occurs through the nuclear pore complexes (NPCs) situated on the nuclear membrane. The NPCs are large proteinaceous structures embedded within the nuclear membrane. The component proteins are arranged to form a central channel approximately 9 nm in diameter that allows diffusion of macromolecules and complexes up to 40 to 60 kDa in size. Transport of larger karyophilic molecules requires expansion of the central channel though an energy dependent process

(Akey, 1990; 1992; Forbes, 1992; Hurt, 1993; Panté and Aebi, 1993; 1994; Agutter and Prochnow, 1994; Hinshaw, 1994; reviewed in Davis, 1995; reviewed in Nigg, 1997; Melchior and Gerace, 1998; Cole and Hammell, 1998; Görlich, 1998).

This movement between the nucleus and cytoplasm is referred to as nucleocytoplasmic transport. Nucleocytoplasmic transport can be separated into the influx of molecules into the nucleus, or import, and the efflux from the nucleus, or export. A variety of proteins such as NHRs and other transcription factors must be transported, after synthesis, into the nucleus in order to associate with DNA and perform their required functions.

1.2.2 Mechanisms of protein transport

Nucleocytoplasmic protein transport has been further described according to the interaction of the protein cargo with intermediate factors during the transport process (reviewed in Nigg, 1990). Two general mechanisms of import have been described. The first mechanism requires expansion of the NPC to allow large proteins and molecules to pass through the central channel. This process, active transport, is energy and temperature dependent, occurs against the concentration gradient, and displays saturation kinetics (Akey, 1990; 1992; Forbes, 1992; Hurt, 1993; Panté and Aebi, 1993; 1994; Agutter and Prochnow, 1994; Hinshaw, 1994; reviewed in Davis, 1995; Görlich and Mattaj, 1996). The second mechanism is the passive diffusion of a small protein through the NPC central channel into the nucleus, where it is retained by binding to an intranuclear component (Peters, 1984). In the case of export, the protein passes through the NPC to the cytoplasm where it may shuttle back into the nucleus or interact with other cytoplasmic factors. Nuclear export is discussed in more detail in Section 1.2.5.

Passive diffusion is a relatively slow process that does not include binding of transporting proteins to structural components of the cell, such as the cytoskeleton, or to receptors aiding in targeting (Peters, 1984). Net accumulation of protein in the nucleus or cytoplasm, in the cases of import and export respectively, is dependent upon the availability of binding sites to which freely diffusing molecules can bind, maintaining the diffusion

gradient. Examples of simple diffusion include import and export of the free catalytic subunit of cAMP-dependent protein kinase, after microinjection into cultured cells (Harootunian *et al.,* 1993); fusion-construct experiments showed that export may be temperature-independent for NLS-bearing proteins (but not for nuclear export signal-, or NES-, bearing proteins) (Fischer *et al.,* 1995; Michael *et al.,* 1995); and egress of β-galactosidase, a normally cytoplasmic protein, occurs in an energy independent fashion when it is fused to a classical NLS (Guiochon-Mantel *et al.,* 1994).

Further studies have suggested that transport of some molecules occurs via diffusion but in a regulated manner, unlike the mechanisms characterised previously (Bustamante, 1992; 1994; Cohen and Paine, 1992; Paine, 1992; 1993; Vancurova *et al.,* 1993; Himpens *et al.,* 1994a; 1994b; Mazzanti *et al.,* 1994; reviewed in Davis, 1995; Jenkins *et al.,* 1998). Facilitated diffusion can be thought of as intermediate between the other two mechanisms, requiring interaction of the cargo protein with cellular factors necessary for signal-mediated import, but not occurring against the chemical gradient. Although energy is not a requirement, and transport proceeds down the chemical activity gradient, transport of some molecules does appear to occur by a mediated process, supporting the existence of a facilitated mechanism (Cohen and Paine, 1992; Jenkins *et al.,* 1998). Molecules that are transported by this process include histone H1 (Breeuwer and Goldfarb, 1990), simian virus 40 (SV40) Vp3 (Dean and Kasamatsu, 1994), and the Vpr protein of human immunodeficiency virus 1 (HIV-1; Jenkins *et al.,* 1998). Interestingly in the case of histone H1, the protein is within the size limits for passive diffusion, at 21 kDa. The absolute criterion that differentiates facilitated transport from active transport is that, in contrast to a facilitated process, active transport occurs against the concentration gradient. For the purposes of this thesis, facilitated and active transport mechanisms are grouped under the term facilitated to enable clearer comparisons to be made between these signal-mediated mechanisms and passive diffusion.

Figure 1.4 displays the three mechanisms of protein import through the NPC into the nuclear compartment, as described in this section. In addition, passive diffusion and signal-mediated nuclear export are also presented (refer Section 1.2.5). Characterisation of import mechanisms is discussed in Chapter Three.

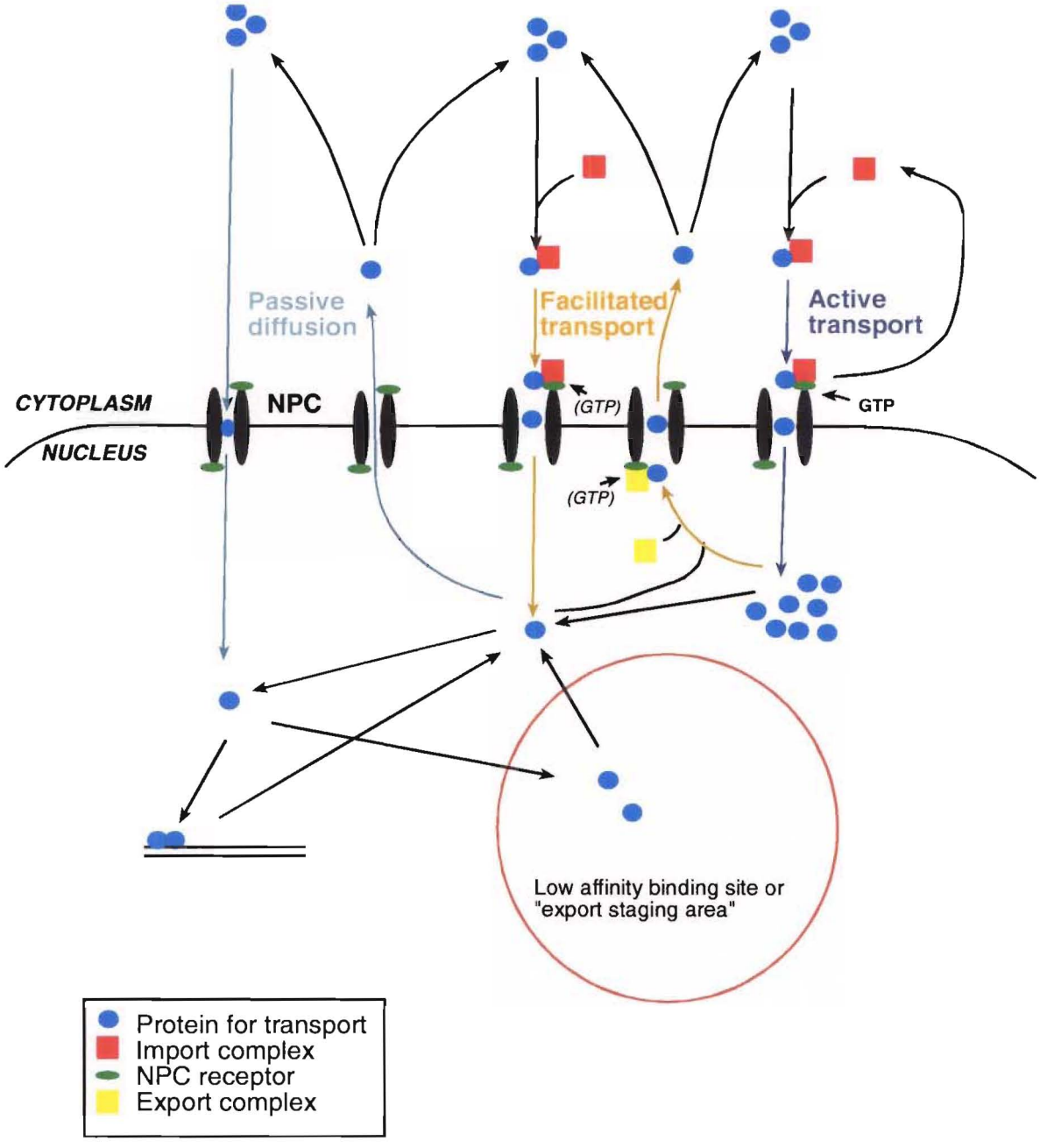


Figure 1.4 - The three mechanisms of nucleocytoplasmic transport. ***See text for details (Sections 1.2.2 and 1.2.3).***

1.2.3 Protein import

Several pathways, which have features in common, have been characterised for signal-mediated protein import. In all these pathways, protein transport through the NPCs can be divided into two steps; firstly, docking of a signal-bearing karyophilic protein complexed with a transport receptor at the NPC, and secondly, translocation through the central channel. The translocation step requires metabolic energy, which may be required to activate the machinery which enlarges the central channel or to move the protein through the channel into the nucleus (Newmeyer and Forbes, 1988; Richardson *et al.,* 1988). Once in the nucleus, a transport receptor dissociates from the transported protein and is recycled to the cytoplasm (reviewed in Nigg, 1997; Ohno *et al.,* 1998).

The best characterised pathway for nuclear import is for those proteins bearing an NLS rich in basic residues, such as the large tumour antigen of SV40 (SV40 T; Lanford *et al.,* 1986) and the RNA-binding protein La (SS-B: Rosenblum *et al.,* 1998). Nuclear localisation signals are discussed in Section 1.2.4. A heterodimeric receptor complex has been isolated for this pathway that is responsible for targeting NLS-bearing proteins to the NPC (see Figure 1.5). Importin α is the component of the receptor complex responsible for NLS binding (Görlich *et al.,* 1994; Weis *et al.,* 1995). Importin β binds importin α and mediates the binding of the transport complex to the NPC (Chi *et al.,* 1995; Görlich *et al.,* 1995a; 1995b; Radu *et al.,* 1995a; 1995b). A small GTP-binding protein related to the Ras family, Ran, was first identified as being responsible for the translocation step in *Xenopus* oocytes (Moore and Blobel, 1993; 1994a; 1994b). The active form of Ran (GTP-bound) is essential for transport of proteins in digitonin permeabilised cells (Melchior *et al.,* 1993; Moroianu and Blobel, 1995). GTP hydrolysis and a Ran activating protein, Rna1p, are required for protein import in yeast cells (Corbett *et al.,* 1995; Schlenstedt *et al.,* 1995). It has been suggested that the metabolic energy requirement for translocation may be supplied by Ran-mediated GTP hydrolysis instead of ATP hydrolysis (Melchior *et al.,* 1993; Cheng *et al.,* 1995; Moroianu and Blobel, 1995; Sclenstedt *et al.,* 1995). Further factors implicated in the importin transport pathway include p10 (Moore and Blobel, 1994b; Paschal and Gerace, 1995), cytoplasmic GTPase activating protein RanGAP, a nuclear localised nucleotide exchange factor RCC1 (Görlich *et al.,* 1996; Izauraude *et al.,* 1997), and the chaperone protein hsp70 (Yang and DeFranco, 1994). Functions and alternative terminologies for the factors involved in this and

other import pathways are detailed in Table 1. Members of the Ran GTP-binding protein superfamily have been implicated in the nuclear import of ribosomal proteins (Rout *et al.*, 1997; Schlenstedt *et al.*, 1997) and proteins involved in RNA processing (Pemberton *et al.*, 1997; Rosenblum *et al.*, 1998), as well as the nuclear export of importin α (Kutay *et al.*, 1997).

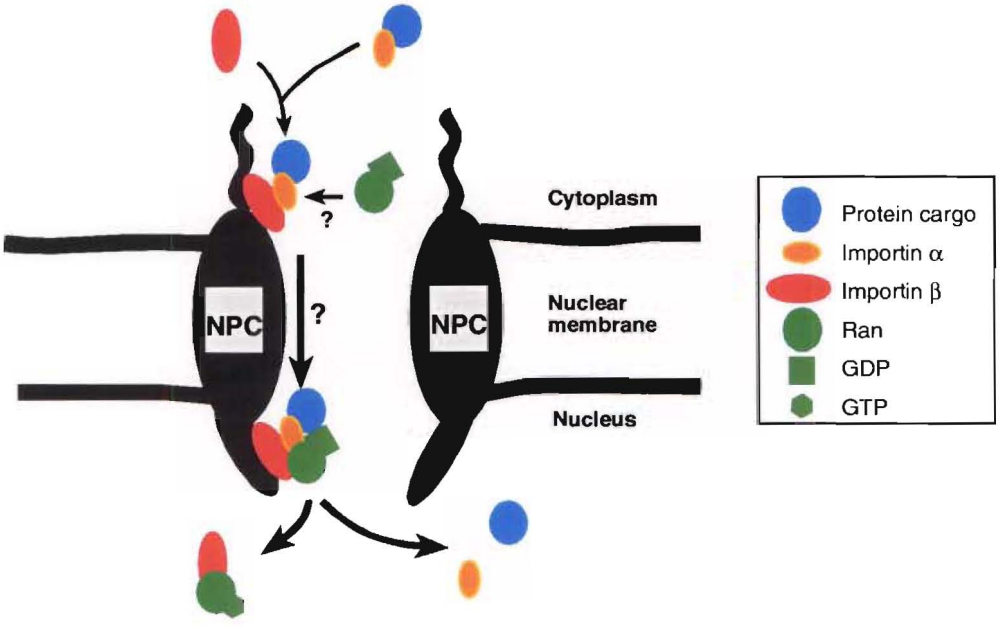


Figure 1.5 - Importin α/β mediated protein import pathway. ***The proposed mechanism for signal-mediated import via the importin α/β complex is modified from Cole and Hammell (1998). Question marks indicate undefined mechanisms. Activation of Ran (by phosphorylation) is shown by interaction with GTP to form RanGDP. Subsequently, GDP is recycled to give GTP by phosphorylation in the nuclear compartment.***

A second unique nuclear import pathway has been described for the RNA-binding protein hnRNP A1. The hnRNP A1 NLS (or M9 sequence) mediates both import and export of the protein (Siomi and Dreyfuss, 1995; Michael *et al.*, 1995). Transportin, a receptor that is distantly related to importin β, has been identified as being responsible for the *in vitro* import of hnRNP A1 (Aitchison *et al.*, 1996; Pollard *et al.*, 1996; Fridell *et al.*, 1997). Transportin mediated transport is also dependent on the presence of Ran (Nakielny and Dreyfuss., 1996).

In contrast to these two pathways, *in vitro* nuclear import assays have shown that although HIV-1 protein Vpr directly engages the NPC, neither transportin nor the importin α/importin β complex are necessary for nuclear import. Further, this process is independent of Ran-mediated GTP hydrolysis (Jenkins *et al.*, 1998).

Table 1 - Terminology of nucleocytoplasmic transport factors.

Term used in this thesis	*Function*	*Localisation*	*Alternative terminology*
Importin α	NLS binding	Cytoplasmic and NPC associated (shuttles)	karyopherin α SRP1α NLS receptor
Importin β	NPC docking of NLS proteins (binds importin α)	Cytoplasmic and NPC associated (shuttles)	karyopherin β p97
Transportin	NPC docking of hnRNP A1 protein-M9 signal	Cytoplasmic and NPC associated (shuttles)	Kap 104
Ran	Translocation (? Role still unclear) Complex dissociation	Cytoplasmic, nuclear, and NPC associated	Ran GTPase Ran/TC4
p10	Translocation (? Role still unclear) Binds to RanGDP and NPC	Cytoplasmic, nuclear, and NPC associated	pp15 Ranip (Ran interacting protein) NTF2 B-2
hsp70	Chaperone	Cytoplasmic and nuclear (shuttles)	hsc70 (cytosolic counterpart of hsp70)
RCC1	Guanine nucleotide exchange factor	Nuclear (chromatin associated)	PRP20 SRM1 MTR1
hRip1	Binds to Rev NES (function unknown)	Nucleoplasm and NPC or NPC only (unclear)	RAB1 YRip1 (homologue?)

1.2.4 Nuclear localisation signals

In the case of import, both facilitated diffusion and active transport pathways require the presence of a karyophilic 'targeting signal' or NLS. Several types of NLSs have been identified. Typically, the NLS is formed from a short stretch of amino acids, approximately eight to ten, which are predominantly the basic residues, lysine or arginine. The most simple NLS is composed of a single stretch of basic amino acids, as in the case of the SV40 T antigen (Kalderon *et al.,* 1984; Lanford *et al.,* 1986).

More complex signals involve several amino acid sequences. A bipartite structure of basic amino acids separated by a stretch of non-basic amino acids forms a second type of NLS, as in the case of nucleoplasmin (Dingwall *et al.,* 1988; Robbins *et al.,* 1991). Two further classes of NLS have been identified that do not conform to the more common stretch of basic amino acids. Dispersal of the amino acids comprising NLSs throughout a large segment of protein suggests a role for secondary and tertiary structures of the protein. Folding of the protein may bring the residues into a more compact form, sometimes referred to as a 'patch signal'. This class of signal has been identified for the U1 small nuclear ribonucleoprotein (RNP)-specific U1A protein (Kambach and Mattaj, 1992), the U2 snRNP-specific U2B protein (Kambach and Mattaj, 1994), and for the Gal4 protein in *Saccharomyces cerevisiae* (Silver *et al.,* 1988). A novel C-terminal glycine-rich domain, referred to as M9, has been implicated in the import of the pre-mRNA/mRNA binding protein hnRNP A1 protein (Siomi and Dreyfuss, 1995). Regardless of its sequence or structural arrangement, a functional NLS is required for the docking step in facilitated and active transport.

Members of the NHR superfamily contain two general types of NLS, constitutive or inducible. Constitutive NLSs are active in all cellular environments, whereas hormone is required for the activation of inducible sequences. This suggests that hormone binding results in an alteration of protein structure allowing NLSs to be presented in an 'open conformation'. Characteristically, NLSs are found in or bordering the DNA binding domain (DBD), the hormone binding domain (HBD), or the hinge region of the receptor. Therefore, it may be difficult to separate out NLS function from other functions of the region, such as DNA binding, which may also be involved in receptor localisation. Steroid hormone receptor NLSs

are complex signals, and in the case of PR and ER, none of the signals for nuclear targeting are sufficient on their own. Table 2 displays the NLSs identified for TR as well as GR, androgen receptor (AR), oestrogen receptor (ER: for the American spelling), and progesterone receptor (PR).

Table 2 - Nuclear localisation sequences present in nuclear hormone receptors.

Receptor	*Number of NLSs*	*Position (a.a) / Designation* [a]	*Observations*	*References*
GR (rat)	Two	497-524 **I** 540-795 (main NLS) **I**	NL1 (hinge) NL2 (HBD)	Picard and Yamamoto, 1987
GR (human)	Two	478-505 **I** 521-777 (main NLS) **I**	NL1 (hinge) NL2 (HBD)	Picard and Yamamoto, 1987 Reviewed in LaCasse and Lefebrvre, 1995
AR (human) (910 a.a.)	Two	608-625 628-634 **I**	NL1 (hinge) NL2 (HBD)	Simental *et al.*, 1991 Jenster *et al.*, 1993
AR (human) (919 a.a.)	Two	580-661	Overlapping signals in the hinge and HBD	Reviewed in LaCasse and Lefebrvre, 1995
ER (human)	Three	256-260 266-271 299-303	Hinge and HBD (none are sufficient NLS singly)	Picard *et al.*, 1990 Ylikomi *et al.*, 1992
PR (rabbit)	Three	638-642/645 (main) **C** 614-618 624-627	NLSs are in hinge and HBD (NL2- Blocked by ATP depletion) (NL3- Weak NLS)	Guiochon-Mantel *et al.*, 1989 Guiochon-Mantel *et al.*, 1991
PR (chicken)	Four	490-506 **C** 477-487 **C** second zinc finger **C** HBD **I**	(NL2- overlaps DBD) (NL3- weak NLS)	Ylikomi *et al.*, 1992
TR (chicken)	One	127-135	Stretch of a.a flanking DBD	Dang and Lee, 1989
TR ($TR\alpha_1$; rat)	One identified in study	134-136	Hinge region	Lee and Mahdavi, 1993
TR ($TR\beta_1$; human)	One identified in study	184-190	Hinge region Lys^{184}-Arg^{185} functionally more important than Lys^{188}-Arg^{189}-Lys^{190}	Zhu *et al.*, 1998
v-ErbA (AEV)	One	370-376	Hinge region	Boucher and Privalsky, 1990 Boucher *et al.*, 1988

[a] The type of NLS is given where known (**I**: hormone inducible NLS; **C**: constitutive NLS).

Cloning of NHRs followed by *in vitro* mutagenesis studies enabled the characterisation of NLSs for GR (Picard and Yamamoto, 1987), PR (Guiochon-Mantel *et al.*, 1989; Ylikomi *et al.*, 1992), ER (Picard *et al.*, 1990), and AR (Simental *et al.*, 1991; Jenster *et al.*, 1993). When NHRs are aligned at the DBDs and a comparison is made, it is observed that sequences homologous to the constitutive PR NLS (a. a. 638-642) are found at the same position (10 amino acids after the last conserved cysteine). The only exception is ER, in which case the signal is located 11 amino acids after the cysteine residue. Four successive clusters of basic amino acids forming an NLS have been identified for chicken PR (Ylikomi *et al.*, 1992). Three constitutive NLSs are located in the zinc finger regions of chicken PR whilst a fourth NLS in the HBD is weakly hormone inducible. In contrast, the two NLSs identified for GR are inducible (Picard and Yamamoto, 1987). Whilst the main NLS was localised to the HBD, the second NLS was inducible and less important in determining receptor localisation. A 20 amino acid signal similar to GR NL1 (in both rat and human) was identified in human AR (Simental *et al.*, 1991). The NLS was located in the hinge region and had the same properties as a bipartite signal, while the second NLS was hormone inducible (Jenster *et al.*, 1993). The three NLSs characterised for ER were not sufficient to act on their own (Picard *et al.*, 1990). The first two NLSs are almost perfectly conserved among species whereas the third is conserved only in charge. Lee and Mahdavi (1993) demonstrated that mutation of the Lys-Arg-Lys residues (134-136 in the hinge region of the receptor) of rat TRα1 was sufficient to disrupt nuclear localisation, suggesting the presence of an NLS at this position. Likewise, a sequence was identified at the same position relative to the DBD in chicken TRα (Dang and Lee, 1989) and in the v-ErbA hinge region (Boucher and Privalsky, 1990; Boucher *et al.*, 1988).

1.2.5 Nuclear export and nucleocytoplasmic shuttling

As nucleocytoplasmic transport can be a bi-directional process, it is important to determine whether both import and export of a particular protein occur. In this situation, the question of whether import and export occur by the same mechanism must also be addressed. Several studies indicate that transport of a range of small proteins is bi-directional; that is, nucleocytoplasmic shuttling occurs. Shuttling proteins identified to date include heat shock

protein 70 (hsp70) (Mandell and Feldherr, 1990), steroid hormone receptors (Guiochon-Mantel *et al.,* 1991), nucleolar proteins (Borer *et al.,* 1989; Meier and Blobel, 1992), heterogeneous nuclear RNP (hnRNP) A1 (Piñol-Roma and Dreyfuss, 1992), and HIV Rev protein (Meyer and Malim, 1994).

Whilst many studies have addressed the process of import, nuclear export has been characterised to a lesser extent. It has been suggested that protein export may be restricted primarily by retention within specific nuclear compartments. In these cases, export may not require a specific signal sequence, but merely proceed via a default pathway which released nuclear proteins are channelled through (Schmidt-Zachmann *et al.,* 1993). Export of the cytoplasmic protein β-galactosidase, which has been artificially introduced by microinjection into the cell nucleus, does require a functional NLS (Guiochon-Mantel *et al.,* 1994), whilst nucleolin export only appears to be temperature dependent (Schmidt-Zachmann *et al.,* 1993). Although PR import is an energy-dependent process, export of receptor continues in the presence of sodium azide (an energy inhibitor) or at low temperatures, suggesting that export occurs via diffusion (Guiochon-Mantel *et al.,* 1991; 1994). Whilst shuttling of some nuclear proteins has been observed, when non-nuclear proteins are microinjected into the nucleus they are unable to be exported (Dingwall *et al.,* 1982; Lanford *et al.,* 1986; Mandell and Feldherr, 1990; Guiochon-Mantel *et al.,* 1991). Although the subject of some controversy, these results suggest that a specific signal (NES) may be required for export of specific proteins (Laskey and Dingwall, 1993; Schmidt-Zachmann *et al.,* 1993). Specific NESs have been identified for protein kinase inhibitor (Wen *et al.,* 1995), hnRNP A1 (m9-NES, Michael *et al.,* 1995), HIV Rev protein (Fischer *et al.,* 1995), and p53 for which export is mediated by a highly conserved leucine-rich NES located in the tetramerisation domain (Stommel *et al.,* 1999). It is unclear whether factors and mechanisms similar to those utilised in import are also used in export: for example, whether NLSs and nuclear export signals are the same. When NLSs of SV40 T antigen and PR are grafted to β-galactosidase, they have the ability to induce shuttling of this normally cytoplasmic protein (Guiochon-Mantel *et al.,* 1994). The presence of a nuclear retention sequence (NRS), that localises the protein to a retention site in the nucleus, has been reported in hnRNP C proteins (Nakielny and Dreyfuss, 1996). The NRS has the ability to override an NES, thereby restricting the protein to the nuclear compartment.

1.3 Aims of investigation and methodological approaches

The aim of the research described in this thesis was to investigate differences in the properties of two TR isoforms, TRα and TRβ, and two variants, v-ErbA and DNA-binding mutant C122A, with respect to subcellular distribution and nucleocytoplasmic transport in two model cell systems, cultured mammalian NIH 3T3 cells and *Xenopus* oocytes. Aspects of the use of these two systems are discussed in Section 1.3.3 (*Xenopus* oocytes) and Section 1.3.4 (cultured somatic cells). Microinjection was used to introduce protein into oocytes and transfection for the introduction of DNA into cultured NIH 3T3 cells. The use of green fluorescent protein tags, utilised in the NIH 3T3 studies, is described in Section 1.3.5.

1.3.1 Comparison of cellular TR isoforms with variant receptors

Members of the NHR superfamily display diverse subcellular distributions and nucleocytoplasmic transport mechanisms. In addition, these distributions and transport mechanisms are still under review due to differences in observations made in varying cell types with different techniques. It has also been observed that localisation of NHRs is not static process and may, rather, be dynamic, involving shuttling of receptor molecules between the cytoplasmic and nuclear compartments.

TR has been reported to be constitutively nuclear in somatic cells (Perlman *et al.*, 1982; Kumara-Siri *et al.*, 1986; Horowitz *et al.*, 1989; Puymirat *et al.*, 1989; Macchia *et al.*, 1992; Lee and Mahdavi, 1993). However, with the advances made in the field of NHR distribution and transport, it seemed likely that the story may not be so simple for TR.

As detailed in Section 1.1.2, there are two common isoforms of TR, designated α and β. Both isoforms are used in this study along with the TRβ DNA-binding mutant variant, C122A, and the viral oncoprotein variant, v-ErbA. The DBD of GR has been shown to be essential for correct localisation and binding to DNA (Sackey *et al.*, 1996). Therefore, use of C122A allows investigation of the role that the TR DBD plays in determining transport characteristics and subcellular distribution of the receptor. Similarly, v-ErbA provides a tool to investigate the effects of an increase in size (an increase from 46.8 kDa in the case of TRα

to 75 kDa) and also the loss of hormone binding ability on nuclear import and export, as well as receptor distribution. Use of oncogenes as tools to investigate cell function is discussed in the following section (Section 1.3.2).

1.3.2 Use of oncogenes as research tools

It is a disconcerting idea that our own cells contain genes with the potential to cause cancer. Nevertheless, some 100 genes, called proto-oncogenes, have been identified in vertebrates that can, when activated, induce tumours. Investigations into the action of these oncogenes must focus on the genetic alterations that produce the uncontrolled cell division and development seen in cancer cells. In normal cells, oncogenes function in conjunction with tumour suppressor genes to control cell growth. Defects, which are inherited or induced by mutagens (such as, radiation, chemicals, or viruses), can potentiate or activate these genes, liberating the cell from its usual regulatory constraints and triggering abnormal cancerous growth (Watson *et al.*, 1987). In addition, activated oncogenes are found widely associated with tumours in humans, suggesting common mechanisms govern transformation by oncogenic retroviruses and other carcinogenic agents (reviewed in Varmus, 1989).

Retroviruses, such as AEV, are the most efficient and rapidly acting oncogenic agents known (reviewed in Teich *et al.*, 1985). The rate of base substitutions in the retroviral genome is approximately one million times that observed in cellular genes (Gojobori and Yokoyama, 1985). This rapid mutation rate is suggested to be due to the lack of proof-reading by the viral polymerase, reverse transcriptase, during reverse transcription of the viral genes (Temin, 1989). Potentially a retrovirus can undergo a series of these mutations before it is subject to selection, and therefore it has been suggested that the evolution of the retroviral oncogene is a mutation-driven process, not unlike the process of cellular proto-oncogene activation (Temin, 1988; reviewed in Weinberg, 1989). Viral oncogenes have proved to be effective tools for investigating cell function by observing changes to normal responses and development.

To date, aberrant expression of thyroid hormone receptors has been implicated in a variety of endocrine and neoplastic diseases, such as small cell lung carcinoma, generalised

thyroid hormone resistance, human colon carcinoma, and hepatocellular carcinoma in mice (Dobrovic *et al.*, 1988; Markowitz *et al.*, 1989; Damm, 1993; Barlow *et al.*, 1994; Yen *et al.*, 1994; Beug *et al.*, 1996; Lin *et al.*, 1997; Fraichard *et al.*, 1997). Likewise, increased response to the steroid hormone, oestrogen, has been linked to the development of breast cancer (Marshall, 1993). As aberrant TR expression and function has the potential to induce abnormal cell growth and development, it is important to define mechanisms induced or repressed by T_3 binding, accessibility to factors that modify the structure and/or activities TR within the nucleus, and the proximity of TR to DNA response elements. This thesis elucidates differences in the distribution and transport properties of TR and v-ErbA. These differences will provide insight into the processes by which vertebrate cells control their differentiation and development, and may aid in comprehension of the events that can lead to transformation of normal cells into tumour cells.

1.3.3 Xenopus oocytes as a model system

Oocytes of the South African clawed frog, *Xenopus laevis*, provide an excellent model system for studying the transport of proteins and other molecules within cells. The use of oocytes is advantageous over cell-free systems as they are able to carry out post-translational modifications and can correctly direct products for protein transport and secretion (Colman, 1984). Exogenous proteins can be expressed in *Xenopus* oocytes through the introduction, by microinjection, of DNA and mRNA (Dawid and Sargent, 1988), and proteins synthesised *in vitro* may also be introduced in this manner (Murdoch and Allison, 1996). Microinjection of oocytes allows direct introduction of cellular components and therefore reconstruction of cell-specific environments *in vivo*, unlike transfected cell cultures. Distribution and transport mechanisms of the introduced molecules can be determined by appropriate assays after a period of incubation. The *src* kinase (Spivack *et al.*, 1984), insulin receptor (Maller, 1987), human epidermal growth factor receptor (Opresko and Wiley, 1990), and ribosomal protein L5 (Murdoch and Allison, 1996) have all been introduced into *Xenopus* oocytes.

Oogenesis in *Xenopus* has been divided into six stages (I to VI), dependent on the morphology of the developing immature egg, or oocyte (Dumont, 1972). Oocytes have nearly

reached their maximum size by stage V (1 to 1.2 mm), when yolk accumulation ceases. Stage VI oocytes are characterised by a distinct unpigmented equatorial band and are ready for ovulation. Large maternal stores of enzymes, storage proteins, and organelles are accumulated during oogenesis (Colman, 1984). The large size of late stage oocytes (V and VI) make *Xenopus* oocytes an attractive option for use in the study of replication, transcription, translation, assembly, and compartmentalisation processes. As proteins, as well as other molecules, can be microinjected into the cytoplasm or nucleus, and both subcellular compartments can be manually dissected, it is possible to study nuclear transport processes quantitatively. The NHRs used in this study were microinjected into either the nuclear or cytoplasmic compartments. After incubation at physiological temperatures, the oocyte nuclei were dissected prior to analysis of the cell fractions.

1.3.4 Cultured mammalian cells as an expression system

Cultured mammalian cells offer an alternative to carrying out investigations in intact organisms. In the early years of cell culture, only masses of excised tissue could be consistently grown on undefined serum or embryonic extracts. Later, groups of individual cells dissociated from tissue by the enzyme trypsin could grow at high cell densities; although single cells could not be induced to grow into clones. By the early 1940's continuous cell lines, established from a single cell, were able to be derived from these cultures as a result of improved techniques and identification of some of the inherent problems of cell culture. Cell lines may be derived from several sources; such as fibroblast, epithelial, or lymphoid tissues. A number of immortal cell lines have been produced from mammalian tumours, as possession of a cancerous phenotype often allows easier adaptation to the conditions of cell culture, although a few 'normal cell' lines have also been established, such as mouse NIH 3T3 cell lines (Copeland and Cooper, 1979). The number of chromosomes in these cell lines is often greater than in normal diploid cells; in the case of NIH 3T3 cells (used in this study), there are 65 chromosomes compared to the normal complement of 40. Mouse NIH 3T3 cells have many of the growth properties associated with normal cells and so can be used for *in vitro* studies of transformation to the cancerous state. The frequency of spontaneous transformation

in NIH 3T3 cells is significantly lower than the rate induced by carcinogenic agents, at one per 10^{-5} to 10^{-6}.

Cultured mammalian cells have been used widely as expression systems for a variety of proteins; such as the NHRs vitamin D receptor (VDR; Barsony *et al.,* 1997) and GR (Carey *et al.,* 1996). Therefore, in this thesis, the results obtained in *Xenopus* oocytes are compared to those in cultured mammalian cells to determine whether the distribution and transport of receptor is peculiar to the oocyte cell type.

Benefits of utilising cultured mammalian cells include the use of completely defined media, of which a wide range of quality controlled product is available commercially. Consequently, cells can be grown in media lacking specific factors (such as sugars or hormones) supplemented with additional agents. Secondly, DNA may also be introduced relatively easily into cultured cells by transfection, which is less labour intensive than microinjection (the method used in this study for introduction of exogenous material into *Xenopus* oocytes). As a negative however, the rate of transfection efficiency varies, dependent upon cell type and transfection reagents and protocols used, whereas microinjection ensures reproducible delivery of exogenous material into a cell. It is also possible to monitor introduction of molecules by microinjection into specific cellular compartments (for example, nuclei) by way of tracking dyes to ensure correctly targeted delivery. Whilst microinjection of cultured cell lines is an established technique, the facilities to adopt this protocol were not available for use in the research described in this thesis.

Although establishment of a cell culture may be a lengthy exercise, potentially an unlimited resource is then available for experimentation whereas maintenance of *Xenopus* colonies is space dependent, and oocyte production is limited in the adult female. In addition, cultures are easily maintained and divided to produce material for experimentation without a need for invasive surgery, as is the case for oocyte removal from the frog.

Lastly, the development of visualisation systems utilising fluorescent tags in living cultured mammalian cells has proved a successful and useful tool, negating a requirement for radioactive labelling and intensive manipulation of antibody detection systems. However, a

comparison of results from a number of systems is important to eliminate artefacts or bias obtained as a result of a particular technique or model system.

1.3.5 Green fluorescent protein tags

Green fluorescent protein (GFP) from the pacific jellyfish *Aequorea victoria* has become an important reporter molecule for monitoring real time gene expression, protein localisation, and protein transport, both *in vivo* and *in situ*. GFP is composed of 238 amino acids and contains a chromophore made up of a Ser-Tyr-Gly tripeptide which undergoes cyclisation and oxidation reactions to become active (Prasher *et al.*, 1992; Cody *et al.*, 1993). Therefore, as GFP fluorescence does not require a substrate or co-factor, it has an advantage over other bioluminescent reporters that do not have an intrinsic chromophore. GFP diffuses throughout the cell and emits bright green light (λ_{max}=509 nm) when excited by blue or UV light (λ_{max}=395 nm, with a minor peak at 470 nm) (Chalfie *et al.*, 1994). Mutagenesis of the GFP cDNA chromophore region has led to the generation of a range of GFPs with different spectral properties or chromophore formation rates (Heim *et al.*, 1994; 1995; Delagrave *et al.*, 1995), referred to as enhanced GFPs, or EGFPs.

GFP has been used successfully in a range of protein localisation and transport studies (Chalfie *et al.*, 1994; Eguchi *et al.*, 1997). When forming a construct with the gene of interest, the gene is fused to the amino or carboxyl terminal end of the GFP gene as determined by the researcher. The protein, when expressed in a living cell, is visualised by fluorescent microscopy. Utilisation of GFP eliminates the need for fixation, cell permeabilisation, and antibody incubation necessary for more traditional techniques. In conjunction with this, GFP is also resistant to photobleaching, which means real time imaging of protein localisation and trafficking is possible.

The GFP used in the studies detailed in this thesis, pEGFP-C1, encodes a red shifted variant of wild type GFP which has been optimised for brighter fluorescence and higher expression in mammalian cells (excitation λ_{max}=488 nm and emission λ_{max}=507 nm). This

variant (referred to as EGFP in this thesis) contains the double amino acid substitution of Phe^{64} to Leu and Ser^{65} to Thr.

Detection of wild type GFP in cultured mammalian cells has proven difficult due to relatively low expression levels. Red shifted mutants of GFP have been used more successfully, showing greater than 15-fold increases in fluorescence (Kitt *et al.,* 1995; Marshall *et al.,* 1995; Stearns, 1995; Cheng *et al.,* 1996). GFP fluorescence is species independent and has been observed in yeast, bacteria, and plants, as well as in a variety of cultured mammalian cells: such as COS-1 (Ogawa *et al.,* 1995); COS-7, NIH 3T3 (Pines, 1995); CHO (Gubin *et al.,* 1997); and HeLa (Pines, 1995; Chatterjee and Stochaj, 1998). GFP has also been effectively used to investigate trafficking and localisation of NHRs, including: AR (Georget *et al.,* 1997), GR (Carey *et al.,* 1996; Htun *et al.,* 1996), and MR (Fejes-Tóth *et al.,* 1998).

1.4 Organisation of thesis

The research contained in this thesis is presented in two sections: firstly, investigation of subcellular distribution of receptor molecules and secondly, characterisation of the mechanism of nucleocytoplasmic transport.

Chapter Two describes the distribution of radioactively-labelled NHRs (TRα, TRβ, v-ErbA, and C122A) between the cytoplasmic and nuclear compartments of *Xenopus* oocytes, after microinjection. In addition, the distributions of TRα and C122A receptors tagged with GFP were examined in living NIH 3T3 cells. The effects of hormone addition on subcellular distribution were assessed in both *Xenopus* oocytes and mammalian cells. Association of *in vitro* synthesised v-ErbA with hsp90 is addressed in Chapter Two. As it has been reported previously that v-ErbA, but not TR, forms an association with this cytoplasmic complex, distribution of v-ErbA may have been influenced by binding to this heat shock protein. Evidence for nuclear binding sites other than DNA sequences alone is presented. The investigation of the subnuclear distribution of receptors, biochemically fractionated from transfected NIH 3T3 cells, is presented.

The processes whereby TR and variants are transported between the subcellular compartments of *Xenopus* oocytes and NIH 3T3 cells are addressed in Chapter Three. A comparison of the mechanisms utilised by the TRα isoform and variants, TRβ isoform, oncoprotein v-ErbA, and DNA-binding mutant C122A, is made in *Xenopus* oocytes. The mechanism of nucleocytoplasmic transport in cultured mammalian cells is presented in Chapter Three and compared with the transport mechanisms defined in *Xenopus* oocytes.

A model is proposed in Chapter Four to account for the observations made during the course of the investigations detailed in this thesis. The interactions of TR with the nuclear machinery and architecture in a variety of cell types are described, placing these results in context with previously reported observations.

Chapter 2

Subcellular Distribution of the Thyroid Hormone Receptor and Variants

2.1 Introduction

2.1.1 Distribution of nuclear hormone receptors

The classical model of steroid hormone action proposes that cytoplasmic pools of receptor are translocated to the nucleus after hormone binding. The liganded receptor associates with the target DNA HRE prior to activation or repression of discrete gene transcription (reviewed in Nigg, 1990). However, by 1986 this model began to be challenged after development of polyclonal and monoclonal antibodies to members of the NHR superfamily (Greene *et al.*, 1980; Logeat *et al.*, 1983; Okret *et al.*, 1984; Sullivan *et al.*, 1986). Antibodies raised against ER, PR, and GR, when used in immunocytochemical studies, overcame the potential problem of artefactual receptor redistribution during cell homogenisation and fractionation studies. Both ER and PR were shown to be localised exclusively to the nucleus *in situ*, even in the absence of hormone (King and Greene, 1984; Perrot-Applanat *et al.*, 1985; respectively). These results supported biochemical fractionation studies, using cytochalasin B-induced enucleation to obtain cytoplast and nucleoplast fractions, which showed unliganded receptor almost exclusively localised to the nucleoplast (Welshons *et al.*, 1984).

The distributions of GR and MR have proven to be somewhat different. It appears that GR is mainly cytoplasmic in the absence of hormone, but nuclear in its presence (Fuxe *et al.*, 1985; Wikström *et al.*, 1987; Rossini and Malaguti, 1994; Akner *et al.*, 1995). However, a subpopulation of GR has been shown to be nuclear in the absence of hormone in nucleoplast/cytoplast studies similar to the ER studies described above (Welshons *et al.*, 1985) and in some immunocytochemical studies (Sanchez *et al.*, 1990; Martins *et al.*, 1991). In some studies, in the absence of hormone, MR was found to be cytoplasmic or both cytoplasmic and nuclear (Lombès *et al.*, 1990; Farman *et al.*, 1991), whilst in the presence of hormone, the receptor was concentrated in the nucleus (Fuxe *et al.*, 1985). More recently, unliganded MR has been described as cytoplasmic, whereas liganded receptor is nuclear in distribution (Robertson *et al.*, 1993; Lombès *et al.*, 1994). Contradictory reports have also been made with regard to VDR. Immunocytochemical studies on chemically-fixed cells showed VDR as exclusively nuclear (reviewed in Barsony *et al.*, 1997), in contrast to microwave fixation studies which revealed a cytoplasmic form of receptor (Barsony *et al.*, 1990). Unliganded AR has been described as nuclear (Husmann *et al.*, 1990; Sar *et al.*, 1990); however, in addition, in cells overexpressing receptor, AR can be cytoplasmic (Simental *et al.*, 1991; Jenster *et al.*, 1993), both cytoplasmic and nuclear (Jenster *et al.*, 1993), or nuclear (Jenster *et al.*, 1993), dependent on cell type. However, in these studies, liganded receptor was always localised to the nucleus.

The distributions of these NHRs as well as TR and v-ErbA, compiled from a variety of experimental studies, are summarised in Table 3. The distributions of TR and v-ErbA are described in the following section.

2.1.2 Distribution of TR and v-ErbA

Although researchers have refocused their attention on the distribution of NHRs, TR and v-ErbA appear to have escaped this renewed interest, except for a recent paper describing the distribution of GFP-TRβ (Zhu *et al.*, 1998).

A significant difference is observed between the subcellular distributions of TR and v-ErbA in mammalian cells. Although all endogenous TR and a subpopulation of v-ErbA appear to be constitutively nuclear, 30-40% of v-ErbA exists cytoplasmically in stable, high molecular weight complexes with heat shock proteins 90 and 56 (Privalsky, 1991; Privalsky, 1992). Qualitative immunofluorescence analyses suggest that stable nuclear accumulation of v-ErbA in cultured mammalian cells requires the integrity of regions of the protein involved in DNA binding. A similar requirement has been observed for the c-*erbA* protein (Boucher and Privalsky, 1990; and references therein). These observations suggest a role for the DNA binding region of the receptors in stable nuclear accumulation of receptor after nuclear import. The DNA-binding mutant (C122A) contains a mutation in one of the two zinc fingers of TRβ, which are present within this domain. Therefore C122A provides a tool to investigate whether DNA provides the sole binding site for TR, or whether alternative sites are available for receptor binding.

It has generally been accepted that TR is constitutively nuclear in the presence or absence of hormone; that is, receptor molecules are bound to TREs (Perlman *et al.,* 1982; Puymirat *et al.,* 1989; Macchia *et al.,* 1990; 1992; Lee and Mahdavi, 1993; Kim *et al.,* 1996). In support of this model, TR molecules are recovered from the nucleus either in tight association with DNA or, after high salt extraction, as monomers (Perlman *et al.,* 1982), and TR has been observed to function as a transcriptional regulator in both the presence and absence of hormone, in mammalian cells (Forman *et al.,* 1988; Forman and Samuels, 1990; Bigler and Eisenman, 1994). TR acts as a ligand-independent activator of gene transcription in yeast cells, *Saccharomyces cerevisiae*, and this expression is increased in the presence of T_3 (Privalsky *et al.,* 1990). In mammalian cells, unliganded receptor can suppress the basal activity of promotors containing TREs (Damm *et al.,* 1989; Sap *et al.,* 1989; Zenke *et al.,* 1990; Baniahmad *et al.,* 1990; 1992). The activator function has been separated from the repressor function (described in Section 1.1.2) by Damm and Evans (1993). At positively regulated TREs, addition of T_3 results in transcriptional activation (Allan *et al.,* 1992; Toney *et al.,* 1993). In the case of negatively regulated TREs, liganded receptors also repress transcription (reviewed in Chatterjee and Tata, 1992).

However, as discussed in Section 1.1.2, some TR-related proteins have been shown to display an extranuclear localisation (Bigler and Eisenman, 1988; Fukuda *et al.*, 1988; Wrutniak *et al.*, 1995; Andersson and Vennström, 1997). Several smaller size cellular TRα proteins occur in chicken erythroid cells, some of which display an extranuclear distribution (Bigler and Eisenman, 1988). A 43 kDa TRα-related protein, with TRE and T_3 binding activities, was found located in the matrix of rat liver mitochondria (Wrutniak *et al.*, 1995). In addition, a significant amount of chicken TR is found in the cytoplasm when transiently expressed at high levels in a rat cell line (Horowitz *et al.*, 1989). This suggests that localisation of receptor may also be dependent on the availability of sufficient receptor sites in the nucleus. Therefore, these findings suggested that the question of the distributions of TR and v-ErbA needed to be revisited.

Table 3 - Distribution of nuclear hormone receptors.

NHR	*Methods*	*Cell type*	*Distribution* [a]	*References*
VDR	Cell fractionation and western analysis	Rat ROS 17/2.8 osteosarcoma	Nuclear (not matrix associated)	Bidwell *et al.*, 1994
	Immunocytochemistry	Osteoblasts	Cytoplasmic nuclear (predominant) (both un/liganded)	Barsony *et al.*, 1990
	GFP-tagged	Human skin fibroblasts	Cytoplasmic/ nuclear	Barsony *et al.*, 1997
v-ErbA	Antiserum raised against v-ErbA	Chicken embryonic erythroid cells	30-40% in cytoplasm	Bigler and Eisenman, 1988 Boucher *et al.*, 1988
TR	High salt extraction, micrococcal nuclease, DNase1	GH_1 cells	Nuclear (DNA associated)	Perlman *et al.*, 1982
	Differential nuclear extraction and [^{125}I]-hormone binding	GC cells	30-50% nuclear matrix associated	Kumara-Siri *et al.*, 1986
	Over expression of chicken c-*erbA*	Rat cell line	Significant in cytoplasm nuclear	Horowitz *et al.*, 1989
	Immunocytochemistry	Hypothalamic DA neurons	Nuclear	Puymirat *et al.*, 1989
	Indirect immunofluorescence	Adult rat liver	Nuclear	Macchia *et al.*, 1992
	Immunocytochemistry	COS cells	Nuclear (un/liganded)	Lee and Mahdavi, 1993
TRβ	GFP-tagged	Living CV1 cells	60% nuclear (unliganded) 85% nuclear (liganded)	Zhu *et al.*, 1998

PR	Immunocytochemistry	Tissue from immature rabbits or castrated guinea pigs	Nuclear (un/liganded)	Perrot-Applanat *et al.*, 1985
	Heterokaryons	L cells	Nuclear plus small percentage shuttling through cytoplasm	Guiochon-Mantel *et al.*, 1991 Kang *et al.*, 1994
ER	Immunocytochemistry	Human mammary and endometrial tumour cells.	Nuclear (un/liganded)	King and Greene, 1984
	Biochemical-cytochalasin-induced enucleation	Rat GH_3 cells	Nuclear (unliganded)	Welshons *et al.*, 1984 Welshons *et al.*, 1985
	Heterokaryons	L cells	Nuclear plus small percentage shuttling through cytoplasm	Guiochon-Mantel *et al.*, 1991 Kang *et al.*, 1994
	Differential nuclear extraction and antibody detection	Female rat liver	Nuclear plus 60% in nuclear matrix (liganded)	Alexander *et al.*, 1987
MR	Immunocytochemistry	Kidney sections	Cytoplasmic (un/liganded)	Lombès *et al.*, 1990 Lombès *et al.*, 1994
	Immunocytochemistry	Rabbit kidney	Nuclear (un/liganded)	Farman *et al.*, 1991
	Immunocytochemistry	Sf9 insect cells (overexpressing) Mouse macrophages (overexpressing)	Cytoplasmic (unliganded) Nuclear (liganded)	Robertson *et al.*, 1993
	GFP-tagged	CHO	Both cytoplasmic and nuclear (unliganded) 100% nuclear (liganded)	Fejes-Tóth *et al.*, 1998
GR	Biochemical studies	Rat GH_3 cells	Nuclear (unliganded)	Welshons *et al.*, 1985
	Immunocytochemistry	CHO	Nuclear (unliganded)	Sanchez *et al.*, 1990 Martins *et al.*, 1991
	Immunocytochemistry	Rat neurons	Cytoplasmic (unliganded) Nuclear (liganded)	Fuxe *et al.*, 1985 Wikström *et al.*, 1987 Rossini and Malaguti, 1994 Akner *et al.*, 1995
	Immunocytochemistry (Nuclear)	Human T24 Human HeLa Rat NRK	Nuclear matrix associated	van Steensel *et al.*, 1995
AR	Immunocytochemistry	Rat/human prostate	Nuclear (unliganded)	Husmann *et al.*, 1990 Sar *et al.*, 1990
	Immunocytochemistry	Transfected (overexpressing)	Cytoplasmic	Simental *et al.*, 1991 Jenster *et al.*, 1993
	Immunocytochemistry	Transfected (overexpressing)	Cytoplasmic/ nuclear	Jenster *et al.*, 1993
	Immunocytochemistry	Transfected (overexpressing)	Nuclear	Jenster *et al.*, 1993

[a] Hormone binding status is indicated when known.

2.1.3 Association of nuclear hormone receptors with the nuclear matrix

Several steroid hormone receptors, including ER, GR, and AR, have been found associated with the nuclear matrix. The nuclear matrix was first described in 1974 by Berenzy and Coffey and has been characterised as the nonchromatin structural protein and ribonucleoprotein scaffolding of the nucleus. This scaffolding is an interconnected meshwork of filaments (nuclear matrix intermediate filaments: NM-IFs) which remains after nuclease, detergent, and high salt extraction of soluble proteins and chromatin (reviewed in Getzenberg, 1994). It is not clear what the protein and RNP composition of the nuclear matrix is. The nuclear matrix includes the lamina proteins, defining the surface of the nucleus, as well as many low abundance nuclear proteins, heterogeneous nuclear RNPs, and matrix-associated DNA regions (Gerace *et al.,* 1978; Kaufman *et al.,* 1983; Fey *et al.,* 1984; 1986; Dworetsky *et al.,* 1992).

More recently, the nuclear matrix has been implicated in regulation of gene expression. The structural framework appears to contribute sites for DNA replication, preferentially associates with actively transcribed genes, and associates with some, but not all, steroid hormone receptors and transcription factors (Berezney, 1991; and references therein). RNA synthesis and pre-mRNA splicing have also been localised to the nuclear matrix (Xing *et al.,* 1993; and references therein). Tissue specificity of nuclear matrix proteins has also been observed (reviewed in Getzenberg, 1994), and two-dimensional electrophoresis methods have been used to identify tumour-specific nuclear matrix proteins in breast, prostate, and colon cancer (Khanuja *et al.,* 1993; Keesee *et al.,* 1994; and references therein). Association of the AML/CBF-α transcription factors with the nuclear matrix is DNA-independent and requires a specific nuclear matrix targeting signal that is distinct from an NLS (Zeng *et al.,* 1997). The presence of nuclear matrix proteins that bind a nuclear matrix targeting sequence and localise growth promoting factors has been reported. For example, the retinoblastoma protein is localised to subnuclear regions associated with RNA processing by nuclear matrix factor p84 (Durfee *et al.,* 1994).

Several NHRs have been shown to associate with the nuclear matrices of a variety of cell lines (Table 3). ER was detected in the nuclear matrices of biochemically fractionated rat liver cells (Alexander *et al.,* 1987). Although GR is predominantly cytoplasmic in the absence of ligand (Fuxe *et al.,* 1985; Wikström *et al.,* 1987; Rossini and Malaguti, 1994; Akner *et al.,* 1995), receptor is localised to the cell nucleus in the presence of hormone, and it has further been shown that GR localises to the nuclear matrix in discrete clusters (van Steensel *et al.,* 1995). Up to 50% of TR has been shown to be associated with the nuclear matrices of rat pituitary tumour cells (GC cells) by [^{125}I]-T_3-binding assays and it has been suggested that this provides a further level of regulation of hormone action (Kumara-Siri *et al.,* 1986). This suggests a role for the nuclear matrix in steroid hormone-mediated control of transcription. However, there is a need to provide supporting evidence by analysis of additional cell lines. All of these findings suggest a role for the nuclear matrix in NHR-mediated control of transcription. Nuclear matrix-TRα associations have not been described in NIH 3T3 cells. Therefore the potential presence of TRα extrachromosomal binding sites, specifically in the nuclear matrix, was investigated during the course of this thesis.

2.1.4 Association of nuclear hormone receptors with hsp90

Heat shock proteins are rapidly synthesised by eucaryotic cells in response to stress, although some members of the heat shock protein family are expressed normally within cells and are involved in protein folding, assembly and subsequent transport (reviewed in Morimoto *et al.,* 1990). Heat shock protein 90 is a 90 kDa molecular chaperone that has been found in a range of organisms from humans to *Xenopus* to bacteria, predominantly as a cytoplasmic dimer (Lindquist and Craig, 1988; Buscher, 1996; reviewed in Meng *et al.,* 1996). Little is known about the mechanism of hsp90 action although a range of associating factors has been identified.

Unliganded GR, AR, and MR are found associated with hsp90 dimers (reviewed in Feldherr and Akin, 1994; reviewed in Brasch and Ochs, 1995; Dittmar *et al.,* 1996). Specifically, hsp90 binds to the HBD of these NHRs (Pratt *et al.,* 1988; Howard *et al.,* 1990; Dalman *et al.,* 1991). PR and ER can also be recovered with the predominantly cytoplasmic

hsp90 (Guiochon-Mantel *et al.*, 1991; Kang *et al.*, 1994). It has been demonstrated that during *in vitro* translation of GR, binding to hsp90 occurs at or near the end of the translation process (Dalman *et al.*, 1989). This association with hsp90 maintains GR in a conformation appropriate for ligand binding, although this may not be the case for other NHRs (Bresnick *et al.*, 1989; Chambraud *et al.*, 1990). Cell-free *in vitro* translation of TR in rabbit reticulocyte lysate does not result in an hsp90-bound form of receptor, whilst generation of GR in the same system results in tight receptor association with hsp90 dimers (Dalman *et al.*, 1990). In this case, TR is translated in a state suitable for DNA binding without transformation by hsp90, whereas GR must be transformed to a DNA binding state. However, GR translated in hsp90-free wheat germ extract does not require this further transformation step (Dittmar *et al.*, 1996). Whilst TR does not associate with hsp90, v-ErbA can be co-immunoprecipitated with hsp90 from mammalian cells if the proteins are chemically cross-linked prior to immunoprecipitation (Dalman *et al.*, 1990; 1991). In addition, RAR, RXR, and VDR are not associated with hsp90 (Dalman *et al.*, 1990; 1991). Associations of various members of the NHR superfamily with hsp90 are summarised in Table 4.

It has been proposed that, although members of the NHR superfamily might differ in the stability of interaction with hsp90, high affinity ligand binding and signalling across the whole family may nevertheless require these interactions (Holley and Yamamoto, 1995). Therefore association with hsp90 may result in repression of DNA binding activity which would directly influence cellular localisation of receptor in the absence of hormone (Dalman *et al.*, 1989; 1990). The *in vitro* transcription/translation rabbit reticulocyte lysate system used to synthesise v-ErbA, in the studies contained in this thesis, include hsp90. Therefore, it was necessary to assess whether v-ErbA was associating with the cytoplasmic heat shock protein, which would prevent nuclear localisation of v-ErbA.

Table 4 - Receptor association with heat shock protein 90.

Receptor	*Association*	*Observations* [a]	*References*
GR	hsp90	Unliganded GR	Dalman *et al.*, 1990 Reviewed Feldherr and Akin, 1994 Reviewed Brasch and Ochs, 1995 Dittmar *et al.*, 1996
AR	hsp90	Unliganded AR	Reviewed Feldherr and Akin, 1994 Reviewed Brasch and Ochs, 1995 Dittmar *et al.*, 1996
MR	hsp90	Unliganded MR	Reviewed Feldherr and Akin, 1994 Reviewed Brasch and Ochs, 1995 Dittmar *et al.*, 1996
ER	hsp90	Unliganded ER in both nucleus and cytoplasm	Guiochon-Mantel *et al.*, 1991 Kang *et al.*, 1994
PR	hsp90	Unliganded PR in both nucleus and cytoplasm	Guiochon-Mantel *et al.*, 1991 Kang *et al.*, 1994
VDR	None		Dalman *et al.*, 1991
RAR	None		Dalman *et al.*, 1991
TR	None		Dalman *et al.*, 1990 Dalman *et al.*, 1991
v-ErbA	hsp90	Requires chemical crosslinking (unlike other receptors)	Dalman *et al.*, 1991

[a] As some experiments were performed *in vitro*, association with cell compartments was not possible and, therefore, is not indicated.

2.1.5 Summary of results

The subcellular distributions of microinjected [^{35}S]-methionine-labelled TRα, TRβ, oncoprotein v-ErbA, and C122A (DNA-binding mutant) were investigated in *Xenopus* oocytes, by polyacrylamide gel electrophoresis and fluorography of proteins extracted from manually dissected oocytes. The properties of TR and variants are summarised in Table 5. Contrary to the dogma of constitutive nuclear localisation, with the exception of v-ErbA, TRα and variants all displayed a similar distribution with only approximately 40% nuclear localisation, following cytoplasmic microinjection. The majority of v-ErbA was sequestered in the cytoplasm, apparently due to an association with hsp90, formed during *in vitro* synthesis of the receptor. A greater percentage of all four receptors (from 62-87%) was retained within the nuclei of nuclear-injected oocytes.

Localisation of EGFP-tagged TRα and C122A was assessed by fluorescent microscopy in a second cell type, mouse NIH 3T3 cells. These receptors, whilst being larger than the untagged receptor molecules, due to the fusion with GFP protein (27 kDa), were expressed effectively in the mammalian system. EGFP-tagged TRα was localised exclusively to the nuclei of cultured NIH 3T3, as reported for native receptors in other cell lines. The ability of receptor to bind DNA appears to be directly involved in receptor distribution, as reflected in the C122A::EGFP distribution, which was 100% extranuclear in NIH 3T3 cells. The subnuclear distributions of TRα (with and without GFP tags for both receptors) were investigated in cultured mammalian cells, by sequential cellular extraction of the NM-IFs, western analysis and immunodetection. C122A::EGFP and C122A distributions were also examined by this sequential extraction method to further characterise cytoplasmic associations and also to determine whether a nuclear population was present that had been undetectable by fluorescent microscopy.

Addition of T_3 induces a shift in TRα, TRβ, and C122A distribution to the nuclei, as well as increased retention of nuclear-injected receptor in *Xenopus* oocytes, but not in cultured cells (for EGFP-tagged TRαand C122A). The distribution of v-ErbA was not significantly affected in oocytes. The implications of the variation between distribution of receptors within and between cell types are discussed.

Table 5 - Properties of TR and variants

Receptor	*Source of Gene*	*Size*	*DNA binding*	*Hormone Binding*
TRα	Rat	46.8 kDa	binding	binding
TRβ	Human	52 kDa	binding	binding
C122A	Human TRβ (modified *in vitro)*	52 kDa	no binding	binding
v-ErbA	Viral (AEV)	75 kDa	binding	no binding

2.2 Materials and Methods

Unless otherwise stated, general laboratory reagents used were obtained from BDH (Poole, England) and electrophoresis reagents were obtained from Boehringer Mannheim (NZ Ltd., Auckland, New Zealand). All chemicals were of Analytical or Molecular Biology grade.

2.2.1 Plasmids and Antibodies

RS-rTRα and RS-v-erbA were gifts from R. Evans (Salk Institute for Biological Studies, La Jolla, CA, USA). In these constructs, rat TRα1 cDNA and the *gag-v-erbA* oncogene were placed under the transcriptional control of Rous sarcoma virus (RSV) long terminal repeats (LTR) (Damm *et al.*, 1989; Thompson and Evans, 1989). RSh-TRβ and RSh-TRβ C>122A were provided by Dr P. Romaniuk (University of Victoria, Victoria, British Colombia, Canada). RSh-TRβ contains the human wild type TRβ1 cDNA and RSh-TRβ C>122A contains an *in vitro*-generated mutant human TRβ1 cDNA (alanine substituted for cysteine at position 122). Both genes are under the control of RSV LTR. (Nelson *et al.*, 1993). Dr P. Romaniuk also provided two constructs containing the same cDNAs but in pGEM3 vectors under the control of T7 RNA polymerase. rTRα1 was a gift from Dr M. Lazar (Lazar *et al.* 1991, University of Pennsylvania School of Medicine, USA) and contains the rat TRα1 cDNA under the control of the T3 RNA polymerase promoter in pBluescript (pBS SK). The construct encoding the gag-v-erbA fusion protein was provided by Dr M. Privalsky (University of California, Davis, CA, USA) and is constructed from two fragments of AEV digested to give the full length gag-v-erbA gene cloned into pGEM-4Z under the control of an SP6 RNA polymerase promoter (Nagl *et al.*, 1997). The ribosomal protein L5 cDNA clone, pSP6-L5, was a gift from Dr W. Wormington (University of Virginia, Charlottesville, VA, USA) and its transcription is under the control of SP6 RNA polymerase (Wormington, 1989).

Fusion plasmids of pEGFP-C1 (Clontech Laboratories, CA, USA) and TRα or C122A were generated by J. M^{c}Grew in the laboratory in which this research was carried out (Dr L. A. Allison's laboratory, University of Canterbury, Christchurch, NZ). These

EGFP-tagged plasmids were used in the localisation and transport studies for TRα and C122A in cultured mammalian cells. In brief, the genes of interest were amplified by polymerase chain reaction (PCR) using primers containing restriction sites from the plasmids RSV-rTRα and pGEM-C122A. Product digested with suitable restriction enzymes was ligated into the multiple cloning site of pEGFP-C1 to produce receptor fused to the C-terminus of EGFP, when expressed *in vivo*. Constructs were sequenced by PCR sequencing to check for correct insertion and amplification errors. Plasmid constructs and primer information are provided in Appendices II and III. EGFP fusion plasmids for v-ErbA and TRβ were not able to be subcloned during the time frame of this thesis, although conventional subcloning techniques through restriction digests followed by ligation, as well as PCR generation of clones, were attempted. It is not clear why the subcloning was unsuccessful, although some of the sequence information provided with the original clones may have contained errors. The EGFP clones are designated TRα::EGFP and C122A::EGFP.

Plasmids were transformed into *Escherichia coli* strain DH5α cells by the method of Brown (Brown, 1991). Plasmids were purified using Qiagen columns (Qiagen, Hilden, Germany), and resuspended in TE, pH 8.0 (10 mM Tris-HCl; 1 mM ethylene-diamine-tetracyclic acid, EDTA). Restriction digests were performed to check integrity of the plasmids isolated.

An antibody, LA038, raised against a synthetic peptide containing residues 58-75 of v-ErbA, was used to detect v-ErbA (Quality Biotech, Camden, NJ, USA; characterised in Freake, *et al.,* 1988). An affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 1-408, representing the full length chicken thyroid hormone receptor, was used to detect TRα and TRβ protein molecules (designated FL-408; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Purified rat monoclonal antibody specific for hsp90 was obtained from StessGen Biotechnologies Corporation (clone 16F1; Victoria, BC, Canada). Mouse IgM monoclonal antibody was used to detect **Nu**clear **M**itotic **A**pparatus protein, or NuMA (Transduction Laboratories, Lexington, KY, USA). Horseradish peroxidase (POD) conjugated goat anti-rabbit IgG secondary antibodies were used to detect the rabbit TRα1 primary antibody during western analyses. Detection of NuMA antibodies was performed with anti-mouse total Ig secondary antibodies conjugated to POD.

2.2.2 *In vitro synthesis of proteins*

In vitro synthesised proteins were prepared from 1 μg circular DNA template (pBS-rTRα1, pGEM-TRβ1, pGEM-C122A, pGEM-v-*erbA*, and pSP6-L5) by coupled transcription/translation in a rabbit reticulocyte lysate system (Promega, Madison, WI, USA) supplemented with SP6, T3, or T7 RNA Polymerase (Epicentre, Madison, WI, USA), as described (Murdoch and Allison, 1996; Nagl *et al.,* 1997). Protein synthesis occurred in the presence of 40 μCi L-[^{35}S]-methionine (1 000 Ci/mmole, *in vitro* labelling grade; Amersham Australia Ltd. or DuPont NEN: Life Technologies Ltd., Auckland, New Zealand).

The approximate amounts of protein synthesised were calculated relative to the amount of control firefly luciferase protein produced during each reaction. The *in vitro* transcription/translation mixture was diluted with sterile ultrafiltered H_2O to give a microinjection concentration of approximately 0.1 ng per 50 nl delivery.

2.2.3 *Surgical removal and microinjection of Xenopus oocytes*

Xenopus laevis (*Xenopus* I, Ann Arbor, MI, USA) were maintained in the Zoology Department at the University of Canterbury. All surgical procedures were approved by the University of Canterbury Animal Ethics Committee. Replicate batches of oocytes were used from different frogs, or after at least a six month recovery period from surgery.

Adult female frogs were anaesthetised by chilling in ice cold water for 30 minutes followed by a further 30 minutes on ice. Sections of ovarian tissue lobes were surgically removed under sterile conditions according to established laboratory protocols (Allison *et al.,* 1991). The frogs were returned to tanks after being sutured with 4/0-coated Vicryl disposable sutures (Ethicon, UK). The tissue was rinsed three times in sterile TNE (abbreviated for: 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA) to remove blood and twice in sterile phosphate-buffered saline, PBS (68 mM NaCl; 1.3 mM KCl; 4.0 mM Na_2HPO_4; 0.7 mM KH_2PO_4; 0.35 mM $CaCl_2$; 0.25 mM $MgCl_2$), to remove EDTA, which could interfere with collagenase activity. To separate the connective tissue from the oocytes, the lobes were chopped into small pieces and incubated in a solution of 30 mg collagenase (Type I, Sigma

Chemical Co., St Louis, MO, USA) in 30 ml 0.1 M sodium phosphate, pH 7.4, with end-over-end rotation (33 rpm) for 20 minutes at room temperature. After separation, the oocytes were rinsed a further two times in PBS and twice in Oocyte Removal Buffer (O-R2: 82.5 mM NaCl; 2.5 mM KCl; 1.0 mM $CaCl_2$; 1.0 mM $MgCl_2$; 1.0 mM Na_2HPO_4; 5.0 mM Hepes; 3.8 mM NaOH). The oocytes were incubated at 18-20°C in culture dishes containing O-R2 until use. The O-R2 was changed daily and any damaged or unhealthy oocytes displaying mottled pigmentation were discarded.

Microinjections were performed according to published methods, with some modification (Allison *et al.*, 1991; 1993). One millimetre O.D. glass capillary tubes (Clark Electromedical, England) were drawn out by a Narishge micropipette puller (Japan) and ground manually to give a needle tip bore of 20 µm. Microinjections were performed using a PV830 pneumatic picopump (World Precision Instruments Inc., New Haven, CT, USA) and micromanipulator (model M3301). The samples were loaded under vacuum and delivered under N_2 gas pressure of 35 psi. Calibration of the amount of sample for delivery was performed by using the calibrated eyepiece of the microscope and alterations in the time (milliseconds) of delivery. Depending on the sample to be injected, a volume of 20 to 50 nl was microinjected. Volume was calculated using the equation:

$$v=\pi d^3/6 \qquad \text{(where 'v' is drop volume and 'd' is drop diameter)}$$

Healthy stage V and VI oocytes were selected based on the classification of Dumont (1972). Stage V oocytes are defined as being 1000 to 1200 mm in diameter, with a pigmented animal pole, whereas Stage VI oocytes are characterised by a distinct unpigmented equatorial band.

2.2.4 Analysis of subcellular distribution in Xenopus oocytes

Oocytes microinjected with *in vitro* translated proteins were incubated for six hours in O-R2 containing 100 µg/ml cycloheximide (Sigma). Whilst cycloheximide inhibits the synthesis of [^{35}S]-labelled proteins *in vitro* from unincorporated [^{35}S]-methionine retained in

the lysate mixture, it does not interfere with protein import in *Xenopus* oocytes (Kambach and Mattaj, 1992). Nuclei were manually removed from the animal hemisphere of the oocyte via a small tear made in the cell membrane with sterile watchmaker's forceps using the method outlined in Colman (1984). To remove any protein adhering to the outside of the nuclear pore complex, Nucleus Isolation Buffer (25 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$; 2 mM dithiothreitol, DTT; 10% glycerol) was used for dissecting oocytes involved in transport studies (adapted from M^{c}Knight *et al.*, 1980 by Parnaik and Kennady, 1990). Adherent cytoplasmic material was removed from the isolated nuclei by pipetting them in and out of an Eppendorf ultramicro-pipette tip. The nuclear fraction was collected separately from the cytoplasmic fraction into microfuge tubes placed on ice (six nuclei and six cytoplasms were separately pooled per sample). Each sample was homogenised in 70 µl Homogenization Buffer (1% Triton X-100; 100 mM NaCl; 20 mM Tris-HCl, pH 7.6, 0.4 mM Pefabloc™ (Boehringer)) using a Gilson pipette. The cytoplasmic sample was further purified by separating the lipid pellicle from each sample by centrifugation at 10 000 × g for 5 minutes at 4°C, in a Jouan MR 14.11 centrifuge (France), and the cleared supernatant was transferred to a new microfuge tube. Five volumes of acetone were added to each sample and protein was precipitated overnight at -80°C. After centrifugation for 15 minutes at 10 000 × g, the protein pellets were air dried before resuspension by vigorous shaking for two hours at room temperature in 20 µl of SDS PAGE-Sample Buffer (2% sodium dodecyl sulfate, SDS; 10% glycerol; 0.6 mM Tris-HCl, pH 6.8; 0.005% bromophenol blue; 0.1 mM DTT).

After denaturation, by boiling for two minutes, samples were placed on ice before separation by discontinuous polyacrylamide gel electrophoresis (PAGE) on 12% polyacrylamide/0.1% SDS gels (SDS-PAGE). Discontinuous polyacrylamide gels consist of a resolving (or separating) lower gel and a stacking (upper) gel. The upper stacking gel is of lower acrylamide concentration and acts to concentrate large sample volumes, which results in better band resolution. Discontinuous gels, 7 cm in length by 8 cm in width, were run in SDS-Running Buffer (959 mM Glycine, 124 mM Tris, 17.3 mM SDS) at 200 volts for 45 to 50 minutes and fixed for between one and 24 hours in gel fixative (25% isopropanol:10% acetic acid). Incubation in an aqueous fluorographic agent, Amplify™ (Amersham Life Sciences, Auckland, NZ), for 15 minutes increased the signal from [^{35}S]-methionine. After

drying on a BioRad gel drier, under vacuum, for two hours at 80°C, gels were visualised by exposure to Hyperfilm-MP autoradiography film (Amersham).

Films were scanned on a Hewlitt Packard Scanjet IICX and quantified by NIH Image, version alpha 9 (Scion Corporation, Frederick, MD, USA). The densities of the bands in the cytoplasmic and nuclear lanes were determined by the Gelplot2 macro. The nuclear density was compared to the sum of the cytoplasmic and nuclear densities to quantify the amount of nuclear localised protein. Nuclear accumulation was expressed as a percentage. Mean sample results from replicate batches of oocytes (n: number of replicate batches presented in figure legends) were analysed for significant difference by the two tailed Student's t-test carried out using Excel 97 (Microsoft® Corporation, Redmond, WA, USA).

2.2.5 *Xenopus oocyte incubations in thyroid hormone*

Oocytes were microinjected with TRα, TRβ, v-ErbA, C122A, or control protein L5, as described in Section 2.2.3. Post-injection, oocytes were incubated in O-R2 supplemented with 100 mM T_3 (Sigma) and 200 μg/ml cycloheximide for six hours at 18-20°C. Protein was isolated from manually dissected oocytes and the distribution of protein in the nuclear and cytoplasmic fractions was analysed according to the standard protocol (Section 2.2.4). Results were compared with control incubations made in the absence of T_3. It has been demonstrated previously in the laboratory that no detectable levels of T_3 are present in *Xenopus* oocytes (Nagl *et al.*, 1995).

2.2.6 *Immunoprecipitation and detection of v-ErbA/hsp90 complexes*

Antibody was bound to pre-swollen protein G-sepharose beads (Pharmacia, Uppsala, Sweden) in NET-2 (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA) at room temperature for two hours with end-over-end rotation. Resin was pelleted for 5 seconds in a microfuge then washed four times in NET-2. The sample for analysis was added to the resin up to a final volume of 0.5 ml with NET-2 (containing 0.1 mM phenylmethylsulfonyl

fluoride, PMSF) and incubated at 4°C for one hour. The resin was collected by centrifugation as before and the supernatant was removed for precipitation in acetone. Prior to addition of 20 µl of SDS-PAGE sample buffer, pellets were washed four times in NET-2. The immunoprecipitates were spun for five seconds in a microfuge, to collect the protein G sepharose beads, after denaturation by boiling for two minutes. Samples were separated on 12% discontinuous polyacrylamide gels, containing SDS, and prepared for exposure to Hyperfilm-MP autoradiography film, as detailed more fully in the Section 2.2.4, or visualised through silver staining of the polyacrylamide gels. Briefly, gels were fixed for one hour in gel fixative prior to detection with the BioRad Rapid Silver Staining protocol (as per the manufacturer's instructions).

The separated protein samples were transferred to Millipore Immobilon PVDF membrane by western blotting in a BioRad Mini Trans-Blot Electrophoretic Transfer Cell. The gel was equilibrated in Western Transfer Buffer (25 mM Tris, 192 mM Glycine, 20% methanol; pH 8.3) before being placed on buffer-soaked 3MM paper (Whatman, Singapore). PVDF membrane was pre-wet briefly in methanol before equilibration in Western Transfer Buffer for two to three minutes. The "transfer sandwich" was assembled by placing the membrane directly upon the gel, and topping with a second piece of buffer-soaked 3MM paper. This sandwich was prepared according to the manufacturer's instructions and transfer was performed overnight in Western Transfer Buffer, at 30 mA and 4°C. Membranes were blotted overnight with shaking in blocking buffer (1% bovine serum albumin and 0.1% Tween 20 in TBS: 10 mM Tris, pH 7.5; 100 mM NaCl). Two washes of 15 seconds each were made in TBS, followed by one for 15 minutes and two washes for five minutes. Subsequently, membranes were incubated at room temperature for one hour in primary antibody (detailed in Section 2.2.1) diluted in blocking buffer to 1: 1000 or at the appropriate dilution determined by titration. Prior to incubation in secondary antibody, membranes were washed, as described above, to remove unbound primary antibody. Membranes were washed again, using the same protocol, after a one hour incubation in secondary antibody conjugated to horseradish peroxidase (POD), diluted to 1:10 000. To initiate the detection reaction, protein blots were placed for one minute in chemiluminescent substrate solution, containing one part ECL Solution I and one part ECL Solution II (Amersham). Blots were drained

briefly and wrapped in a single layer of plastic wrap prior to exposure to Hyperfilm-MP autoradiography film for between 30 seconds and five minutes.

2.2.7 Mammalian cell culture

NIH/3T3 cells are derived from NIH Swiss mouse embryo cultures and are highly contact-inhibited (American Type Culture Collection, Rockville, MD, USA). A tissue culture facility was set up and protocols were prepared for culturing NIH 3T3 cells. Tissue handling was performed in a Class II Biological Safety Cabinet (BH2000 series; Clyde Apac, Australia) with rigorous aseptic techniques. Cells were maintained at 5% CO_2:95% air in a humidified Napco 5415 IRCO_2 system incubator (Precision Scientific, Chicago, IL, USA). Cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 10% calf serum (CS) and 1% penicillin-streptomycin stock (Stock: 10 000 units penicillin and 10 000 µg streptomycin/ml), as required (Life Technologies, Gaithersburg, MD, USA), at 37°C under 5% CO_2:95% air in the humidified incubator. Cells were reseeded by trypsinisation weekly with 0.1% trypsin and media replaced twice weekly, or as required. Cell stocks, in Cell Freezing Medium (Life Technologies), were frozen and maintained in liquid nitrogen according to the manufacturer's instructions.

2.2.8 Transfection of NIH 3T3 cells

NIH/3T3 cells were reseeded into a variety of vessels, dependent upon the experiments in which they were to be used. Cell stocks were maintained in Nunc 25 cm^2 filter top flasks (Nalge Nunc International, Rochester, NY, USA). For the purpose of GFP experiments, cells were seeded onto Lab-Tek two or four well tissue culture chamber slides (Nunc). Large quantities of cells were required for the nuclear matrix preparations, therefore, cells were seeded in 100 mm culture dishes (Nunc). Twenty four hours after reseeding into DMEM/10% CS, cells at 50-60% confluence were transfected with plasmid DNA and lipofectamine reagent, in Opti-MEM I Reduced Serum Medium, according to the manufacturer's instructions (Life Technologies). Transfection conditions were optimised for

DNA and reagent concentrations, and transfections were performed for between 16 and 20 hours at 37°C and 5% CO_2:95% air. Media was replaced 24 hours post transfection. Living cells were washed gently in Dulbecco-modified PBS (D-PBS: 2.7 mM KCl; 1.5 mM KH_2PO_4; 136.9 mM NaCl; 8 mM Na_2HPO_4) and examined 48 hours post transfection to visualise EGFP fusion proteins. Cells for nuclear matrix-intermediate filament isolation (NM-IF) analysis were prepared as detailed below (Section 2.2.11).

2.2.9 Visualisation of EGFP labelled proteins in cultured mammalian cells

The transfected cells on chamber slides were rinsed gently in D-PBS to remove any calf serum that might otherwise autofluoresce. Cells being maintained at 4°C were washed in ice-cold D-PBS and maintained on ice for all stages except microscopic examination. Cells incubated at 37°C were maintained in a portable incubator (TYPE). The slide chambers were disassembled according to the manufacturer's instructions and cells were mounted in fresh D-PBS under number 1 coverslips. Cells were maintained at the temperature incubations were performed at, with D-PBS being added as required to prevent dehydration of the preparations. Slides were examined on a Leitz Orthoplan fluorescence microscope under both bright field and fluorescent conditions (Blau BG38/4mm; Blau BG12/2mm; K495; K470/490). Observations were also made in combined light conditions to better enable localisation of fluorescence to cellular compartments. Photography was performed with a Leitz Orthomat camera and Fujichrome Provia 400 Daylight slide film, in all light conditions. Replicate slides were examined and distributions of EGFP proteins were recorded qualitatively. Distribution and the percentage of transfection were calculated for each experiment, prior to compilation of the results for each experimental type.

2.2.10 T_3 addition and absence in cultured mammalian cells

DMEM culture medium was stripped of thyroid hormone (T_3) according to the protocol of Yen *et al.* (1994). In brief, DMEM containing 10% calf serum was mixed with 5% AG1-X8 resin (BioRad) for 12 hours at 4°C. The medium was decanted, before addition of fresh resin to 5%, and a second incubation was carried out for 12 hours at 4°C, with

stirring. The media was immediately filtered by peristaltic pump into a sterile bottle before storage at 4°C.

Cells were seeded and transfected with EGFP and fusion plasmids in the standard manner. Twenty four hours post transfection, the cell medium was replaced with T_3-stripped DMEM, or supplemented T_3-stripped DMEM containing a range of T_3 concentrations (10^{-9} M, 10^{-6} M, 10^{-3} M). A 100 mM T_3 stock was prepared in 100% ethanol, from which subsequent dilutions were made into 100% ethanol. Stocks were maintained at -20°C until use. Cells were examined for fluorescence after a further 24 hours incubation at 37°C and 5% CO_2:95% air.

2.2.11 Isolation of nuclear fractions and nuclear matrix intermediate filaments

NIH/3T3 cells were seeded into 100 mm culture dishes (Nunc) to give 50-60% confluence after 24 hours. Cells were transfected with the EGFP fusion vectors specified in Section 2.2.1, as detailed in Section 2.2.8. The increased size and possible non-specific affinities of the EGFP fusion proteins for cellular compartments could influence the nuclear distribution of the tagged receptors. Therefore, the non-EGFP "source plasmids", also described in Section 2.2.1, were included as controls for EGFP-tagged receptors. Forty eight hours post-transfection, cells were harvested according to an established protocol (He *et al.*, 1990). Ten plates were placed on ice at once and the cells were harvested. After removal of culture medium, cells were rinsed in ice-cold D-PBS. Plates containing ice-cold D-PBS were scraped using a disposable cell scraper (Nunc). The detached cells were transferred to a conical tube on ice. Cell detachment was completed in ice-cold D-PBS, which was transferred between plates prior to scraping. The NIH 3T3 cells were collected by centrifugation at 880 × g for five minutes at 4°C, in a Jouan CR412 (M4 swing out rotor) centrifuge, prior to separation of individual cell fractions in various buffers. Fractions were collected after each centrifugation step described below. Pellets were resuspended in CSK buffer (100 mM NaCl; 300 mM sucrose; 10 mM Pipes, pH 6.8; 3 mM $MgCl_2$; 1 mM EGTA; 0.5% Triton X-100; 1.2 mM PMSF), which dissolves phospholipids and soluble proteins whilst leaving the cytoskeleton intact. The supernatant containing the phospholipids and soluble proteins was

collected by centrifugation as before. The cytoskeleton and polyribosomes were removed from the remaining cell fractions by resuspension in RSB-Majik buffer (10 mM Tris, pH 7.4; 10 mM NaCl; 3 mM $MgCl_2$; 1% Tween 40; 0.5% deoxycholate; 1.2 mM PMSF) and subsequent centrifugation. Chromatin was digested in digestion buffer (50 mM NaCl; 300 mM sucrose; 10 mM Pipes, pH 6.8; 3 mM $MgCl_2$; 1 mM EGTA; 0.5% Triton X-100; 1.2 mM PMSF), containing 400 µg/ml DNase 1 and 50 µg/ml RNase A (Sigma Types II and IIIA from bovine pancreas, respectively) for 20 minutes at room temperature on a tilting shaker. One half volume of 0.5 M ammonium sulphate was added to release the digested chromatin and associated histones. After 10 minutes centrifugation at 880 × g and 4°C, the isolated nuclear matrix intermediate filament (NM-IF) pellet was separated from the chromatin fraction and resuspended in digestion buffer without additives. Small aliquots of the NM-IF and other collected fractions were snap frozen in liquid nitrogen and stored at –80°C.

2.2.12 Western analysis of differentially extracted proteins

One volume of 2×SDS/PAGE-sample buffer was added to 16 µl of sample (from NM-IF preparations). Samples were denatured, by boiling for two minutes, prior to loading on 12% discontinuous polyacrylamide gels, containing SDS. Gels were run at 20 mA for approximately one hour and transferred to PVDF membrane by Western transfer as described in Section 2.2.6.

The quantities of protein loaded on the polyacrylamide gels (contained within the 16 µl sample) were determined by microassays performed with a BioRad Protein Assay Kit with a serum albumin standard, according to the manufacturer's instructions. The reaction absorbance was measured at 540 nm on a Ultrospec II spectrophotometer (Pharmacia LKB Biochrome Ltd., Cambridge, England). The amount of protein contained in each sample is shown in Table 8.

2.3 Results

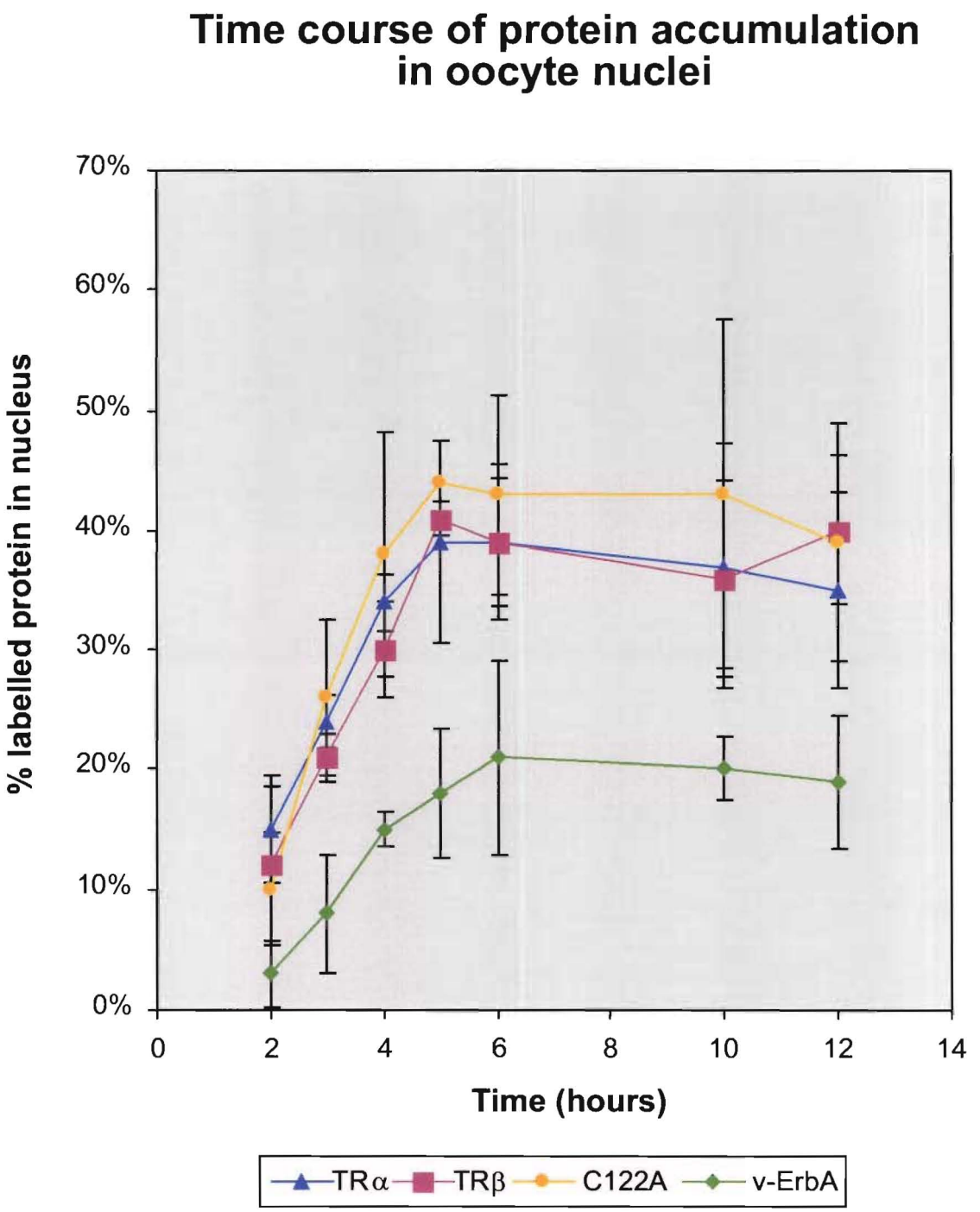
The results in this chapter are presented according to the cell system used for each experiment. Distribution studies carried out in microinjected *Xenopus* oocytes are described in Sections 2.3.1, 2.3.2, and 2.3.4. These sections present the results for the investigation of TRα, TRβ, C122A, and v-ErbA import kinetics, determination of the subcellular distributions of these receptors, and the effect of hormone incubation on receptor distribution, respectively. Results of hsp90 and v-ErbA association studies are given in Section 2.3.6. Sections 2.3.3, 2.3.5, and 2.3.7 present the results obtained in cultured NIH 3T3 cells. Subcellular distributions for EGFP-tagged TRα and C122A are presented in Section 2.3.3, and T_3 incubations in Section 2.3.5. The results for fractionation studies performed in TRα, TRα::EGFP, C122A, and C122A::EGFP transfected cells are contained in Section 2.3.7. Results for both *Xenopus* oocyte and cultured NIH 3T3 cell systems are summarised in Table 6 and Table 7, respectively. These tables also contain the probability values for Student's t-tests carried out between data sets.

2.3.1 Microinjected [^{35}S]-labelled TR and variants accumulate in Xenopus oocyte nuclei

To determine the optimal time at which to perform distribution and transport assays, the kinetics of nuclear import for TRα, TRβ, C122A, and v-ErbA were assayed over time. Steady state was reached at approximately six hours post-injection (Figure 2.1). Further analyses of receptor distribution were carried out between five and six hours, after the steady state was reached. The difference in receptor levels at each time point (indicated by the error bars) is due to variability among batches of oocytes. The amount of protein to be used in each injection was determined by titration. The range of protein concentrations assessed included 50, 100, and 200 picograms per 50 nanolitres injection volume (data not shown). The concentration of 100 picograms per injection was selected for future experiments as this produced a strong signal, when pooled fractions from six oocytes were extracted and analysed by SDS-PAGE and fluorography. No difference was observed in protein distribution between 100 and 200 picrograms.

Figure 2.1. ***The nuclear accumulation of microinjected TR and variants in <u>Xenopus</u> oocytes.***

Approximately 100 picograms of *in vitro* synthesised [^{35}S]-methionine labelled protein were cytoplasmically-microinjected per oocyte. Oocytes were incubated at 18-20°C over the time intervals indicated on the x-axis of the graph. Nuclei were manually dissected from the oocytes and proteins were isolated from six pooled nuclear or cytoplasmic fractions, according to the respective microinjections. TRα, TRβ, C122A, v-ErbA, or L5 protein was separated from oocyte proteins by electrophoresis on 12% discontinuous polyacrylamide gels containing SDS, followed by autoradiography. The percentage of nuclear accumulation was determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of replicate batches (**n**) of six pooled oocytes (TRα: n=8; TRβ: n=4, C122A: n=4; v-ErbA: n=9). Standard error of the means is indicated by the bars.


Time course of protein accumulation in oocyte nuclei
% labelled protein in nucleus
70%
60%
50%
40%
30%
20%
10%
0%
0
2
4
6
8
10
12
14
Time (hours)
TRα
TRβ
C122A
v-ErbA

2.3.2 Subcellular distribution of TRα, TRβ, C122A, and v-ErbA in Xenopus oocytes

DNA binding is an important determinant in the localisation and tight binding of GR in the nucleus (Sackey *et al.,* 1996). As TR has been reported to be constitutively bound to DNA, it follows that integrity of the DBD may play a role in receptor localisation. In addition, TR and v-ErbA deletion mutants for the NLS domains, which are located close to the DBD (see Table 2), were found to be localised to the cytoplasm (Dang and Lee, 1989; Lee and Mahdavi, 1993). TR variant C122A contains a mutation in the first zinc finger of TRβ, and is therefore unable to bind DNA. Thus, the distribution of DNA-binding mutant C122A was compared with wild type TRα, TRβ, and v-ErbA to assess whether DNA binding is necessary for stable nuclear accumulation of receptor.

Unexpectedly, the distributions of TRα, TRβ, C122A, and v-ErbA observed in *Xenopus* oocytes (Figure 2.2) were distinctly different from previous reported findings made in cultured somatic cells for TRα (Perlman *et al.,* 1982; Kumara-Siri *et al.,* 1986; Horowitz *et al.,* 1989; Puymirat *et al.,* 1989; Macchia *et al.,* 1992; Lee and Mahdavi, 1993) and v-ErbA (Bigler and Eisenman, 1988; Boucher *et al.,* 1988). Although some TR-related proteins have been observed to have cytoplasmic forms, TR has not previously been shown to have a cytoplasmic subpopulation (as discussed in Sections 1.1.2 and 2.1.2).

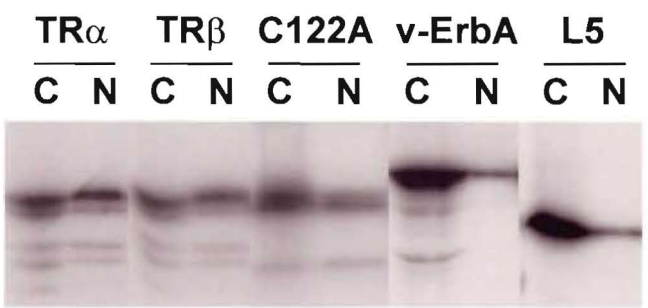
Thirty nine percent of cytoplasmically-injected TRα was localised to the nuclei of *Xenopus* oocytes after a five to six hour incubation (Figure 2.2, A and B). Similarly, TRβ and C122A were distributed between the cytoplasm and nucleus, with 39% and 41% localised to the nucleus, respectively (Figure 2.2, A and B), suggesting that DNA binding is not essential for nuclear localisation.

Figure 2.2. ***The distribution of cytoplasmically-injected TR and variants in Xenopus oocytes.***

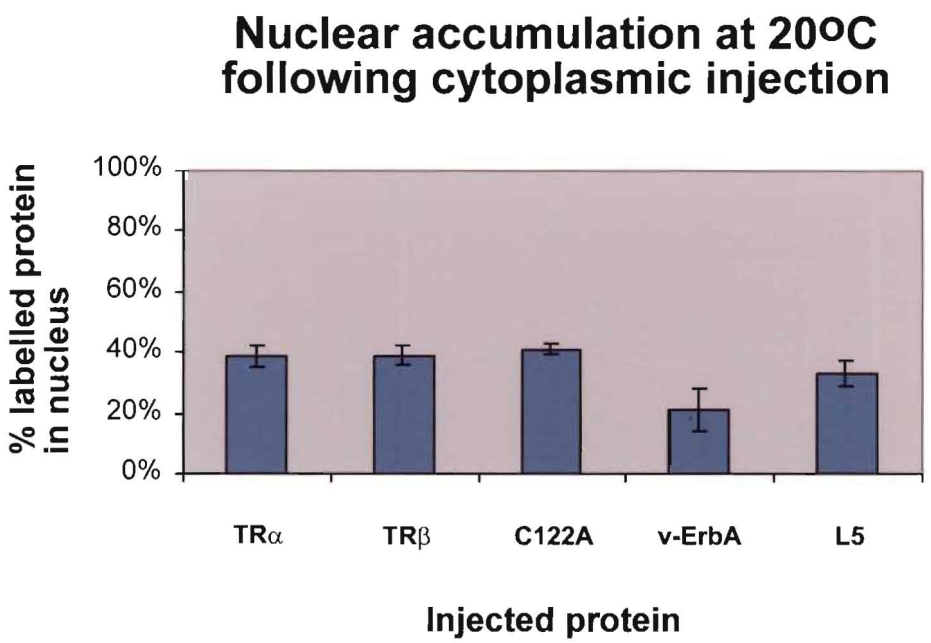
A. *In vitro* synthesised [^{35}S]-methionine labelled protein was introduced into either the cytoplasmic or nuclear compartment of each oocyte by microinjection, as detailed in Figure 2.1. After a five hour incubation at 18-20°C, nuclear accumulation of TRα, TRβ, C122A, v-ErbA, and L5 was analysed as described in the legend of Figure 2.1. A representative autoradiogram is shown. (**C**: cytoplasmic fraction; **N**: nuclear fraction).

B. The graph shows nuclear accumulation results for replicate experiments (**n**) of cytoplasmically injected L5 (n=4), TRα (n=8), TRβ (n=4), C122A (n=4), and v-ErbA (n=9).

A


TRα
TRβ
C122A
v-ErbA
L5
C N C N C N C N C N

B


Nuclear accumulation at 20°C following cytoplasmic injection
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
TRα
TRβ
C122A
v-ErbA
L5
Injected protein

In contrast to the constitutive nuclear localisation of TR reported for somatic cells (Perlman *et al.,* 1982; Kumara-Siri *et al.,* 1986; Horowitz *et al.,* 1989; Puymirat *et al.,* 1989; Macchia *et al.,* 1992; Lee and Mahdavi, 1993), 30-40% of v-ErbA has been shown to be cytoplasmic in mammalian cells (Privalsky 1991; 1992). The distribution of v-ErbA in *Xenopus* oocytes also varies from that reported previously. In oocytes, 21% of the receptor was distributed to the nucleus and 79% was cytoplasmic (Figure 2.2, A and B). Ribosomal protein L5 was used as a control protein in this study as import kinetics have been well characterised in Dr L. A. Allison's laboratory (Murdoch and Allison, 1996). As expected a subpopulation of L5 was localised to the nucleus (33%; Figure 2.2A).

The subcellular distribution of receptor molecules, microinjected into the nuclei of *Xenopus* oocytes, was also determined after five to six hours (Figure 2.3). Nuclear-injected TRα was retained in the nucleus to a greater extent than TRα injected into the cytoplasm: 62% (Figure 2.3, A and B) compared with 39% (Figure 2.2, A and B). A greater proportion of nuclear injected TRβ, C122A, v-ErbA, and L5 molecules was also retained in oocyte nuclei (74%, 72%, 87%, and 68%, respectively; Figure 2.3, A and B) compared with nuclear localisation after cytoplasmic injection (39%, 41%, 21%, and 33%, respectively; Figure 2.2, A and B).

2.3.3 Distribution of EGFP-tagged TRα and C122A in cultured mammalian cells

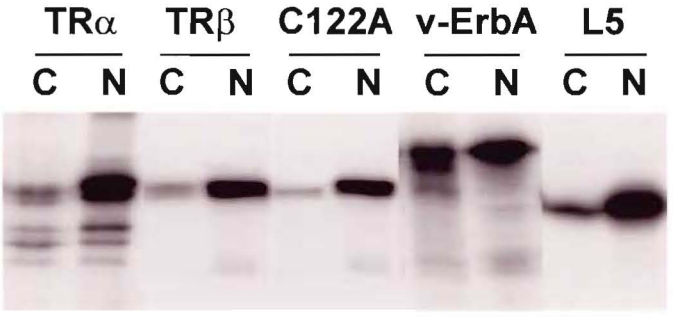
GFP has been shown to autofluoresce in a variety of cultured mammalian cells: such as COS-7, HeLa, and NIH 3T3 (Pines, 1995). GFP has also been effectively used as a tag to investigate transport and localisation of a variety of NHRs; including GR (Carey *et al.,* 1996; Htun *et al.,* 1996), AR (Georget *et al.,* 1997), and MR (Fejes-Tóth *et al.,* 1998).

Figure 2.3. ***The distribution of nuclear-injected TR and variants in Xenopus oocytes.***

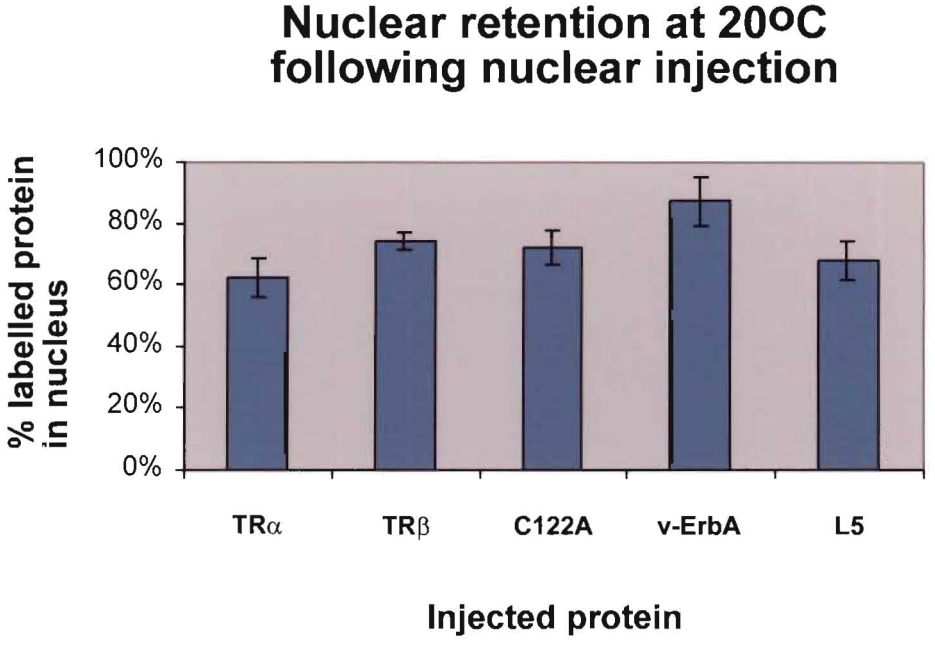
A. [^{35}S]-labelled TRα, TRβ, C122A, v-ErbA, or L5 was introduced into oocyte nuclei by microinjection. After five to six hours incubation at 18-20°C, protein was isolated from oocytes, manually dissected into cytoplasmic (**C**) and nuclear (**N**) fractions, and analysed, as described in Figure 2.1. A representative autoradiogram is shown.

B. The percentage of labelled protein in the oocyte nuclei from replicate experiments (**n**) was quantified by densitometry of the autoradiograms produced in the experiments described above. (TRα: n=8; TRβ: n=3, C122A: n=3; v-ErbA: n=5; L5: n=4)

A


TRα
TRβ
C122A
v-ErbA
L5
C N C N C N C N C N

B


Nuclear retention at 20ºC
following nuclear injection
% labelled protein
in nucleus
100%
80%
60%
40%
20%
0%
TRα
TRβ
C122A
v-ErbA
L5
Injected protein

The α isoform of TR has long been described as constitutively bound to DNA in cultured mammalian cells (Perlman *et al.,* 1982); that is, no cytoplasmic subpopulation of the receptor has been identified (as discussed in Section 2.1.2). This raises the possibility that DNA binding may play a key role in nuclear retention of these receptors. However, contrary to this notion, no significant difference was seen in oocytes between the distributions of TR and the DNA-binding mutant, C122A. To determine whether these two proteins are similarly distributed in living, cultured mammalian cells, red-shifted EGFP fusion proteins were used to visualise the localisation of TRα and C122A in these cells. Wild type EGFP protein was used as a control. Transfection efficiencies are recorded in Table 7.

As expected, EGFP was present throughout the cell, as visualised in Figure 2.4. In support of previously reported distributions (Perlman *et al.,* 1982; Puymirat *et al.,* 1989; Macchia *et al.,* 1992; Lee and Mahdavi, 1993), EGFP-tagged TRα was localised to the cell nuclei of NIH 3T3 cells in all observed experiments. A representative example of this localisation is presented in Figure 2.4, whilst all data are summarised in Table 7. In contrast to EGFP-tagged TRα, and to the results obtained in *Xenopus* oocytes, EGFP-tagged C122A was completely excluded from the nuclear compartment of NIH 3T3 cells (Figure 2.4).

2.3.4 T_3 hormone increases nuclear accumulation of TR in Xenopus oocytes

This series of experiments aimed to address whether stable nuclear accumulation of TR in *Xenopus* oocytes is dependent upon the HBD. Results are summarised in Table 6. Whilst, TRα, TRβ, and C122A display the ability to bind hormone, mutations have rendered v-ErbA unable to bind T_3 at physiological concentrations.

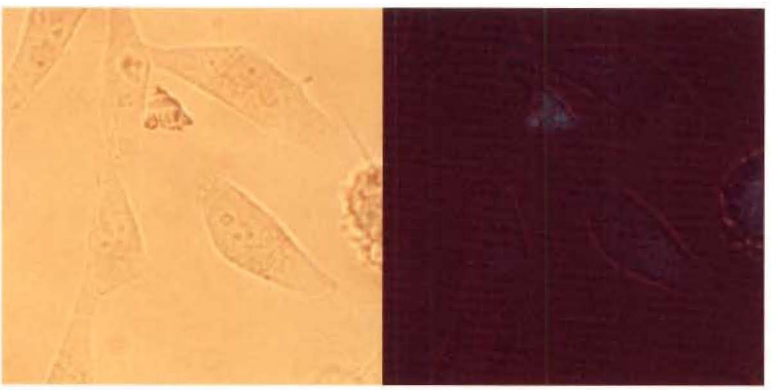
Addition of T_3 to O-R2 incubation buffer altered the distribution of cytoplasmically injected TRα. Hormone binding induced a shift of receptor from the cytoplasmic compartment to the nucleus. Analysis by Student's t-test indicated a significant increase ($p<0.001$) from 39% to 51% nuclear TRα (Figure 2.5, A and B). This shift was also observed for nuclear injected TRα, with 85% of TRα retained in the nucleus compared to 62% ($p<0.001$; Figure 2.5, A and B).

Figure 2.4. ***The subcellular distribution of EGFP-tagged TRα and C122A in cultured mammalian cells.***

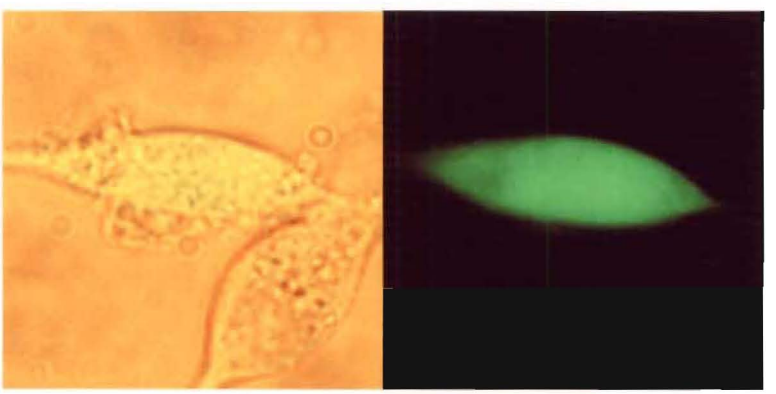
Plasmid constructs containing TRα or C122A fused to EGFP were transfected into NIH 3T3 cells, cultured to 50% confluence. Transfection medium was replaced by complete medium after 24 hours incubation at 37°C and 5% CO_2:95% air. Cells were analysed after a further 24 hours incubation. Living cells were rinsed with D-PBS prior to visualisation with a fluorescent microscope, a combination of bright-field and epifluorescence, as well as with epifluorescence only (as shown). Control transfections with EGFP and with no EGFP were performed. (**TRα**: EGFP-tagged TRα; **C122A**: EGFP-tagged C122A)

37°C incubations

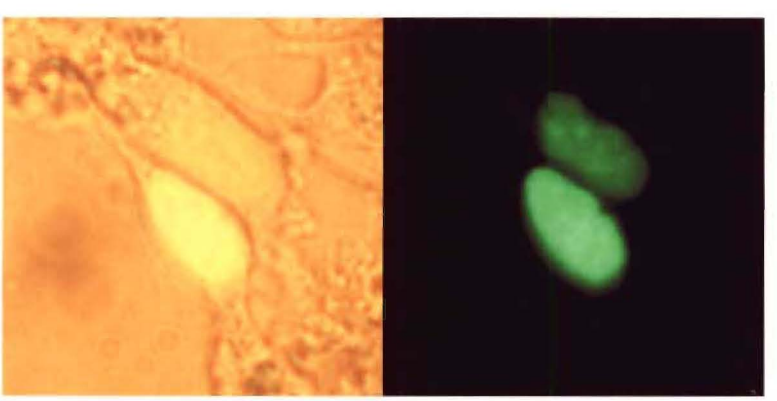
CONTROL
(pEGFP minus)



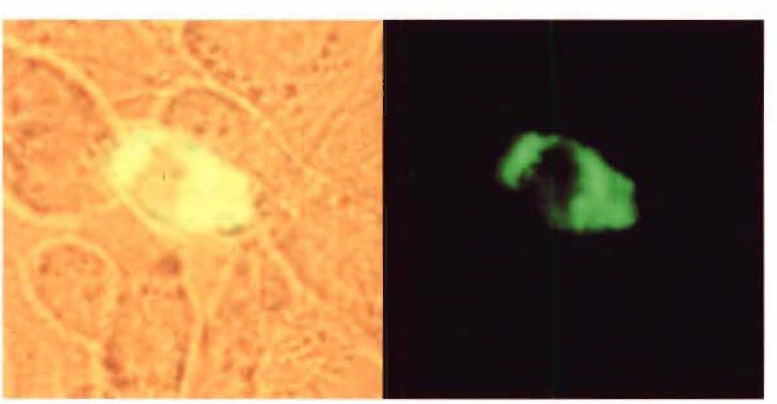
pEGFP



TRα



C122A



Conversely, no significant increase ($p>0.1$) in v-ErbA nuclear localisation was observed when oocytes were incubated in T_3 (Figure 2.6). However, as such a small percentage of receptor is localised to the nuclei of oocytes incubated at physiological conditions, it would be difficult to assess even a one to two fold increase in nuclear receptor after incubation in T_3.

As seen for TRα, there was an increase in C122A and TRβ receptor accumulation in nuclei of oocytes incubated in T_3 (Figure 2.7, A and B). However, this was smaller in magnitude and was significant for TRβ at the 95% confidence level ($p=0.03$). In the case of TRβ, T_3-incubation of oocytes increased nuclear accumulation from 39% to 45%, compared with a shift from 41% to 46% nuclear for C122A. The shift for C122A was only significant at the 90% level of confidence ($p<0.1$).

In addition to incubations at physiological temperatures, oocytes microinjected with either TRα or v-ErbA were incubated at 0-4°C, in the presence or absence of T_3 hormone. A significant shift ($p<0.001$) of TRα to the nuclei of chilled, T_3-incubated oocytes is shown in Figure 2.5 (A and B). The observed change is an increase in nuclear protein from 40% to 48%. Addition of T_3 at 0-4°C does not affect v-ErbA distribution (Figure 2.6, A and B), although visualisation of such a shift may be difficult, as discussed above.

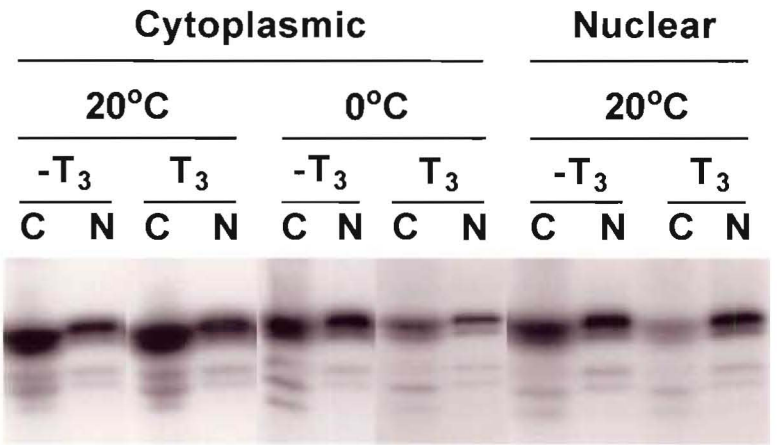
A control incubation of ribosomal protein L5 in T_3 did not result in an increase of L5 in oocyte nuclei (Figure 2.7, A and B). In fact, a slight reduction in nuclear L5 was observed which skews the t-test results. As L5 does not bind T_3 hormone, this confirms that the change in TR distribution is attributable to hormone binding, and is not due to a non-specific effect.

Figure 2.5. ***The effect of T_3 incubation on TRα distribution in Xenopus oocytes.***

A. Approximately 100 picograms of *in vitro* synthesised [^{35}S]-methionine labelled TRα were introduced into each oocyte by microinjection. Oocytes were incubated at 18-20°C or 0-4°C (as indicated) in O-R2, or in O-R2 supplemented with 100 mM T_3 (as indicated), for five to six hours prior to manual dissection of the oocytes into cytoplasmic (**C**) and nuclear (**N**) fractions. Protein was isolated from six pooled nuclear or cytoplasmic fractions and separated from oocyte proteins by electrophoresis on 12% discontinuous polyacrylamide gels containing SDS, followed by autoradiography. A representative autoradiogram is shown.

B. The percentages of nuclear accumulation from the experiments described in (**A**) were determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of replicate batches (**n**) of six pooled oocytes. Standard error of the means is indicated by the bars. (TRα[20°C/-T_3]: n=8; TRα[0°C/-T_3]: n=4; TRα[20°C/T_3]: n=8; TRα[0°C/T_3]: n=4; TRα[20°C/-T_3/nu.]: n=8; TRα[20°C/nu./T_3]: n=3) (**T_3**: thyroid hormone; **-T_3**: minus thyroid hormone; **nu.**: nuclear injections)

A


Cytoplasmic
Nuclear
20°C
0°C
20°C
-T3
T3
-T3
T3
-T3
T3
C N C N C N C N C N C N

B

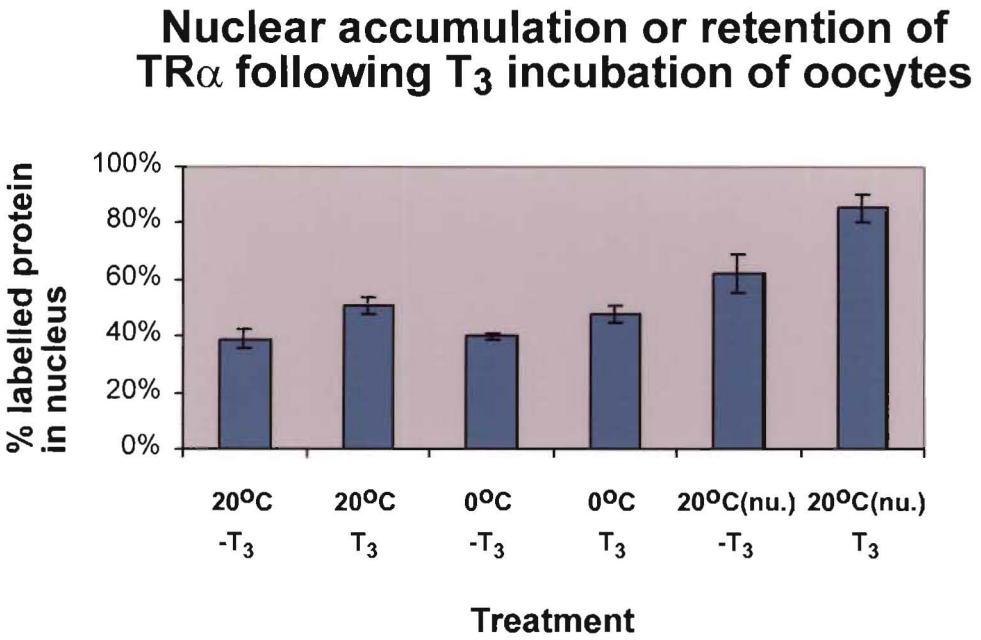
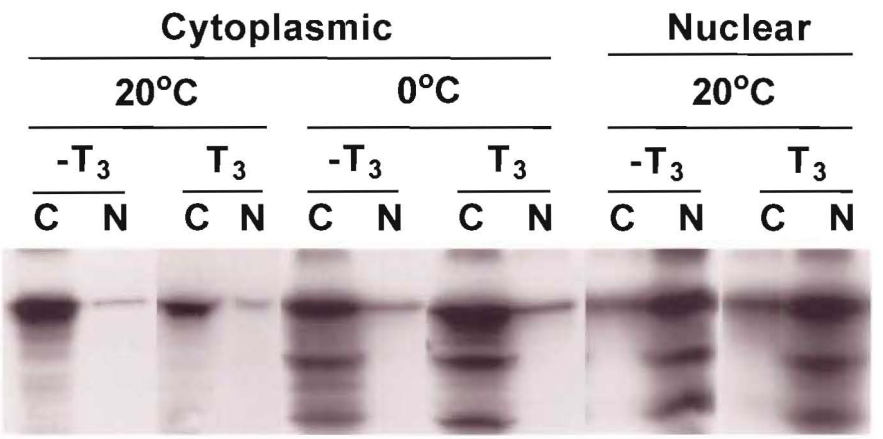

Nuclear accumulation or retention of TRα following T3 incubation of oocytes
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
20°C -T3
20°C T3
0°C -T3
0°C T3
20°C(nu.) -T3
20°C(nu.) T3
Treatment

Figure 2.6. ***The effect of T_3 incubation on v-ErbA distribution in Xenopus oocytes.***

A. Approximately 100 picograms of *in vitro* synthesised [^{35}S]-methionine labelled v-ErbA were introduced into each oocyte by microinjection. Oocytes were incubated at 18-20°C or 0-4°C (as indicated) in O-R2, or in O-R2 supplemented with 100 mM T_3 (as indicated), for five to six hours prior to manual dissection of the oocytes into cytoplasmic (**C**) and nuclear (**N**) fractions. Protein was isolated from six pooled nuclear or cytoplasmic fractions and separated from oocyte proteins by electrophoresis on 12% discontinuous polyacrylamide gels, followed by autoradiography.

B. The percentages of nuclear accumulation from the experiments described above were determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of replicate batches (**n**) of six pooled oocytes. Standard error of the means is indicated by the bars. (v-ErbA[20°C/-T_3]: n=9; v-ErbA[0°C/-T_3]: n=4; v-ErbA[20°C/T_3]: n=4; v-ErbA[0°C/T_3]: n=4; v-ErbA[20°C/-T_3/nu.]: n=5; v-ErbA[20°C/nu./T_3]: n=4) (**T_3**: thyroid hormone; **-T_3**: minus thyroid hormone; **nu.**: nuclear injections)

A


Cytoplasmic
Nuclear
20°C
0°C
20°C
-T3
T3
-T3
T3
-T3
T3
C N C N C N C N C N C N

B

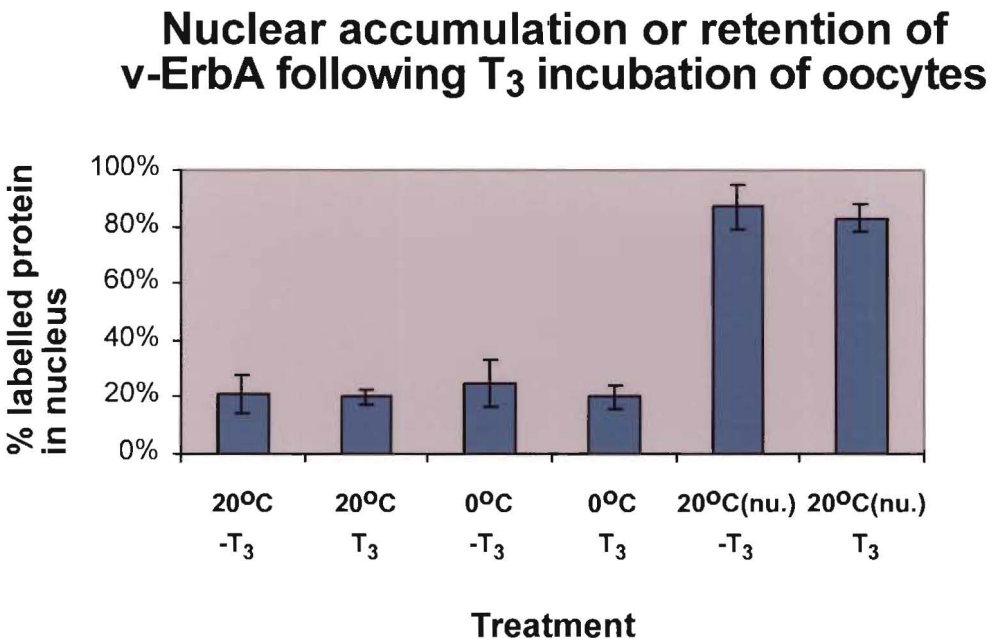
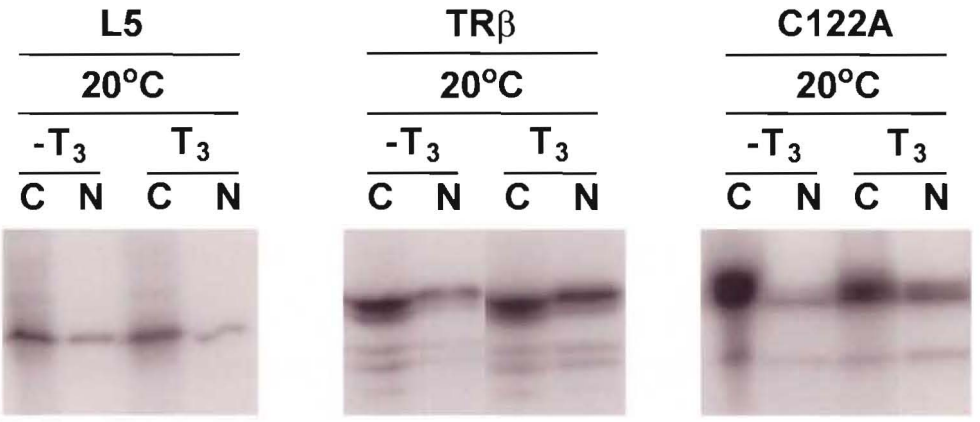

Nuclear accumulation or retention of v-ErbA following T3 incubation of oocytes
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
20°C -T3
20°C T3
0°C -T3
0°C T3
20°C(nu.) -T3
20°C(nu.) T3
Treatment

Figure 2.7. ***The effect of T_3 incubation on TRβ, C122A, and L5 distribution in <u>Xenopus</u> oocytes.***

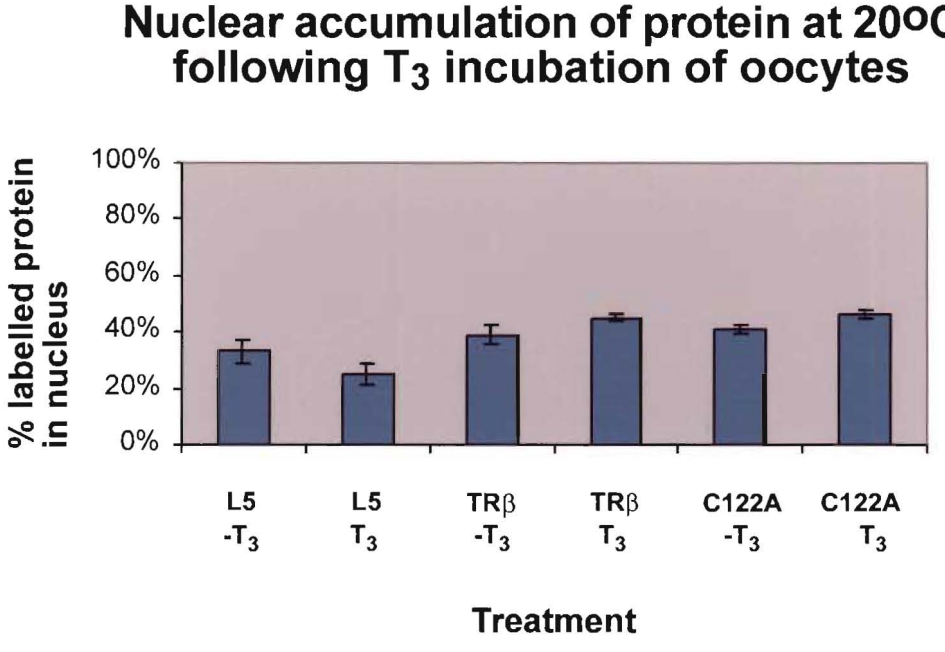
A. Approximately 100 picograms of *in vitro* synthesised [^{35}S]-methionine labelled TRβ, C122A, or L5 were introduced into each oocyte by microinjection. Oocytes were incubated at 18-20°C or 0-4°C (as indicated) in O-R2, or in O-R2 supplemented with 100 mM T_3 (as indicated), for five to six hours prior to manual dissection of the oocytes into cytoplasmic (**C**) and nuclear (**N**) fractions. Protein was isolated from six pooled nuclear or cytoplasmic fractions and separated from oocyte proteins by electrophoresis on 12% discontinuous polyacrylamide gels, followed by autoradiography.

B. The percentages of nuclear accumulation from the experiments described above were determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of three to six replicate batches (**n**) of six pooled oocytes. Standard error of the means is indicated by the bars. (L5[20°C/-T_3]: n=4; L5[20°C/T_3]: n=3; TRβ[20°C/-T_3]: n=4; TRβ[20°C/T_3]: n=2; C122A[20°C/-T_3]: n=4; C122A[20°C/T_3]: n=2) (**T_3**: thyroid hormone; **-T_3**: minus thyroid hormone; **nu.**: nuclear injections)

A


L5
20°C
-T3
T3
C N C N
TRβ
20°C
-T3
T3
C N C N
C122A
20°C
-T3
T3
C N C N

B


Nuclear accumulation of protein at 20°C following T3 incubation of oocytes
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
L5 -T3
L5 T3
TRβ -T3
TRβ T3
C122A -T3
C122A T3
Treatment

2.3.5 *Hormone does not affect localisation of EGFP-tagged TRα or C122A in cultured cells*

Addition of T_3 to culture medium induced a nuclear shift in TRα, TRβ, and C122A distribution in *Xenopus* oocytes (Section 2.3.4). As more nuclear retention sites appear to become available to hormone-bound receptors in oocytes, it was of interest to know whether this is the case in cultured mammalian cells. C122A, although DNA binding deficient, retains its hormone binding ability (Section 1.1.4). Therefore, a shift in distribution from 100% cytoplasmic (Figure 2.4) might be predicted to occur with variation in hormone concentrations. Results for the hormone incubation studies described in this section are summarised in Table 7.

Cultured NIH 3T3 cells were incubated in medium stripped of T_3, or supplemented with hormone at the following concentrations: 10^{-3} M; 10^{-6} M; 10^{-9} M. Synthesis of protein was arrested, by addition of cycloheximide, four hours before observation of receptor distribution. No alteration in EGFP-tagged C122A or TRα distribution was observed in response to the T_3 treatments used (Figure 2.8, A and B). T_3 incubations of EGFP transfected cells were performed, with no observable differences from control cells incubated in the absence of hormone (Figure 2.8C). Transfection efficiencies are recorded in Table 7.

2.3.6 *v-ErbA binds hsp90 from rabbit reticulocyte lysate*

It has been proposed that complexing of NHRs with hsp90 renders the receptor unable to bind DNA, by sequestering the receptor in the cytoplasm (Dalman *et al.,* 1990). In this way, hsp90 directly influences the localisation of the NHR in a hormone absent cellular environment, by preventing nuclear import of the receptor. Whilst v-ErbA associates with hsp90, TR does not form a heterocomplex with the heat shock protein (Dalman *et al.,* 1989; 1990). Therefore, it is important to determine whether the v-ErbA synthesised *in vitro* during the course of this study associates with hsp90 present in the reticulocyte lysate mixture.

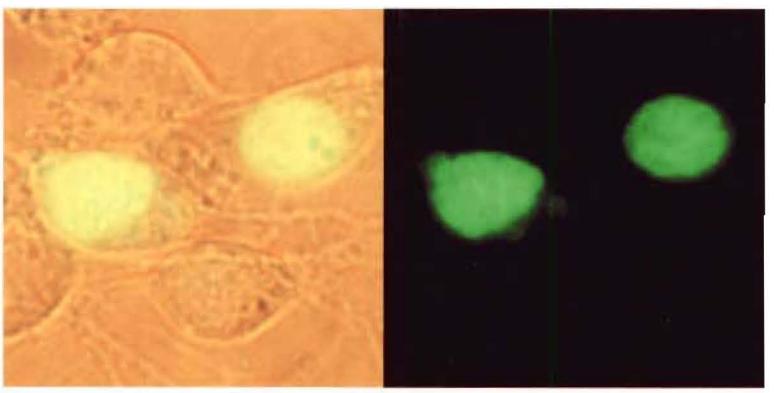
Figure 2.8. ***The effect of T_3 incubation on TRα::EGFP, C122A::EGFP, and EGFP distribution in cultured mammalian cells.***

NIH 3T3 cells were transfected with TRα::EGFP (**A**), C122A::EGFP (**B**), or EGFP (**C**) and incubated as described in Figure 2.4. Transfection medium was replaced by complete medium after 24 hours incubation at 37°C and 5% CO_2:95% air. Twenty four hours after incubation in complete medium, cells were washed with D-PBS. Cycloheximide (50 μg/ml) was added to the complete medium for thirty minutes. This medium was replaced with T_3 minus medium containing 50 μg/ml cycloheximide or medium supplemented with 10^{-3}M, 10^{-6} M, or 10^{-9} M T_3 for three hours incubation at 0-4°C. Cells were washed with D-PBS and visualised under a fluorescent microscope with a combination of bright-field and epifluorescence, as well as with epifluorescence only.
(**TRα:** EGFP-tagged TRα; **C122A**: EGFP-tagged C122A; **T_3**: thyroid hormone; **-T_3**: minus thyroid hormone)

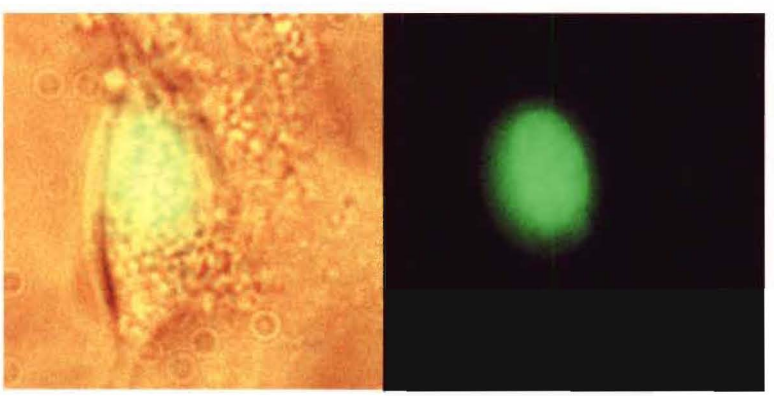
A

T_3 incubations

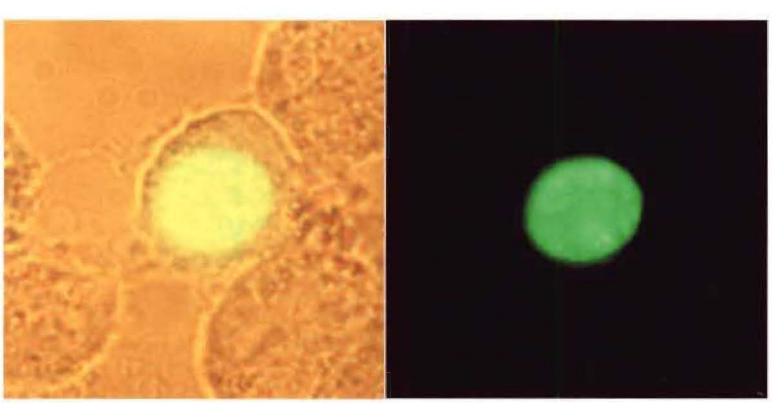
*TR*α
T_3 minus



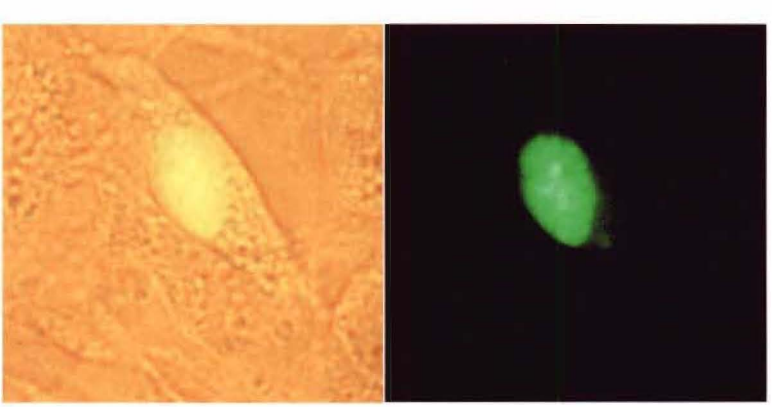
10^{-9} M T_3



10^{-6} M T_3



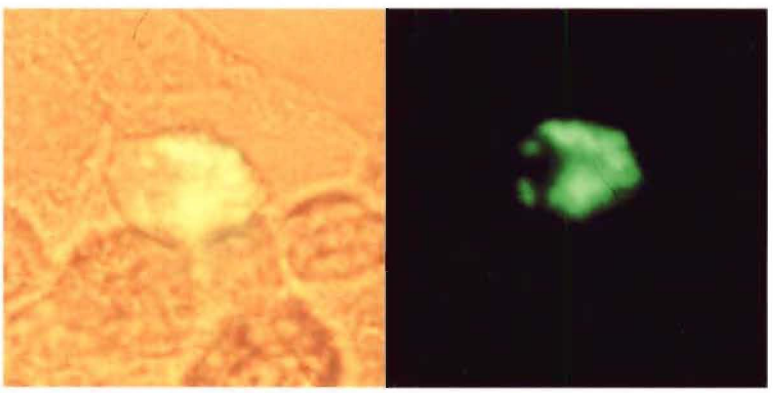
10^{-3} M T_3



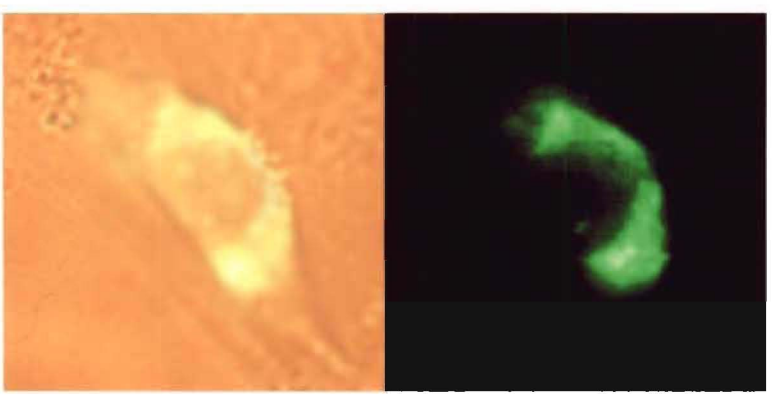
B

T_3 incubations

C122A
T_3 minus

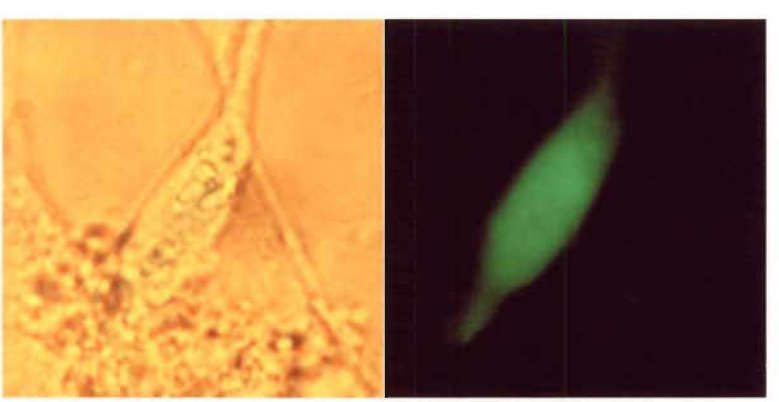


10^{-3} M T_3

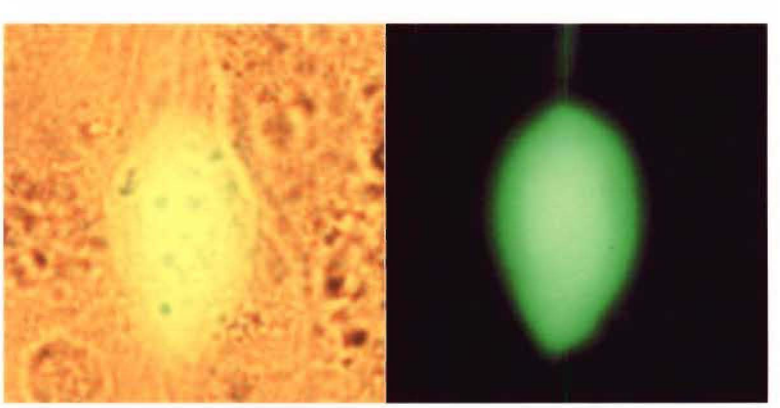


C

pEGFP
T_3 minus



10^{-3} M T_3



The rationale for this set of experiments was that if v-ErbA complexes with hsp90 during synthesis, then the receptor would be effectively localised in an immobile complex in the cell cytoplasm after cytoplasmic microinjection into oocytes. Therefore, if v-ErbA were isolated by specific antibodies, any associated hsp90 would also co-precipitate from the rabbit reticulocyte lysate translation mixture. This hsp90 could then be detected in turn by a second hsp90-specific antibody.

Monoclonal antibodies raised against v-ErbA or antibodies against hsp90 were used to immunoprecipitate [^{35}S]-methionine-labelled v-ErbA from the translation mixture. When the immunoprecipitate was separated by PAGE, the coimmunoprecipitate (isolated by way of anti-hsp90) and immunoprecipitated v-ErbA (from anti-v-ErbA isolation) was visualised by autoradiography (Figure 2.9A) or silver staining (results not shown).

Serial dilutions of anti-hsp90 or anti-v-ErbA are shown in Figure 2.9A. At all dilutions, v-ErbA was immunoprecipitated by antibody. However, hsp90 was not detectable by silver staining (not shown).

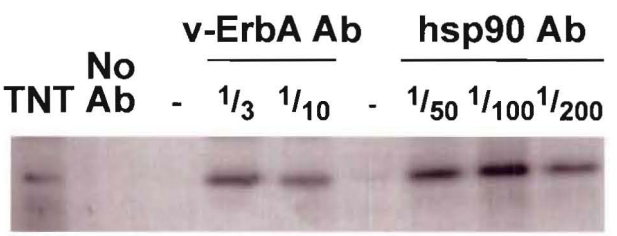
When the separated proteins were transferred to membranes by western transfer, anti-hsp90 antibodies detected protein bands running at the expected molecular weight of 90 kDa in the relevant lanes (Figure 2.9B). The second lane contains the anti-v-ErbA immunoprecipitate (from anti-v-ErbA dilution $^{1}/_{3}$, Figure 2.9A), whereas the lane labelled $^{1}/_{50}$ is the same as that isolated by anti-hsp90 in Figure 2.9A. The immunoprecipitates were compared to a positive control lane of transcription/translation mixture (TNT) that contains rabbit reticulocyte lysate, in which hsp90 has been previously detected (Dalman *et al.,* 1990). These results show that the v-ErbA synthesised *in vitro* is already complexed with hsp90 prior to being introduced into *Xenopus* oocytes.

Figure 2.9. ***Hsp90 associates with v-ErbA in rabbit reticulocyte lysate.***

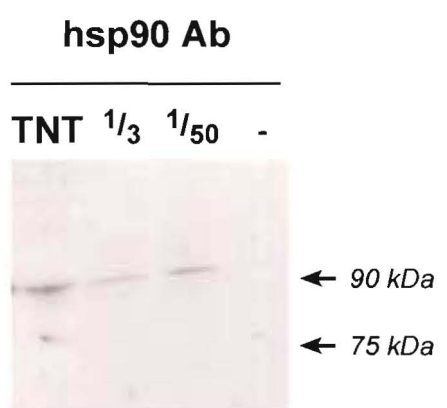
A. Hsp90 and v-ErbA were immunoprecipitated from rabbit reticulocyte lysate with various concentrations of anti-v-ErbA monoclonal antibodies. The immunoprecipitates were separated by discontinuous SDS-PAGE and [^{35}S]-methionine labelled v-ErbA was visualised on X-ray film. (**TNT**: 1 µl of v-ErbA transcription coupled translation mix, positive control; **No Ab**: no antibody, negative control; **-**: empty lane; **1/3, 1/10**: dilutions of v-ErbA Ab; **1/50, 1/100, 1/200**: dilutions of anti-hsp90 **Ab**; Ab: antibody). Replicate experiments were performed (**n**=3).

B. Immunoprecipitates from above were transferred to PVDF membrane by western transfer techniques and hsp90 was detected on the blots by hsp90-specific antibodies. (**TNT**: 8 µl; **1/3**: v-ErbA Ab immunoprecipitate from **A.**; **1/50**: hsp90 Ab immunoprecipitates from **A.**; **-**: empty lane). (**n**=1)

A


v-ErbA Ab
hsp90 Ab
No
TNT Ab
-
1/3
1/10
-
1/50
1/100
1/200

B


hsp90 Ab
TNT
1/3
1/50
-
90 kDa
75 kDa

2.3.7 Subnuclear distribution of wild type and EGFP fusion receptors

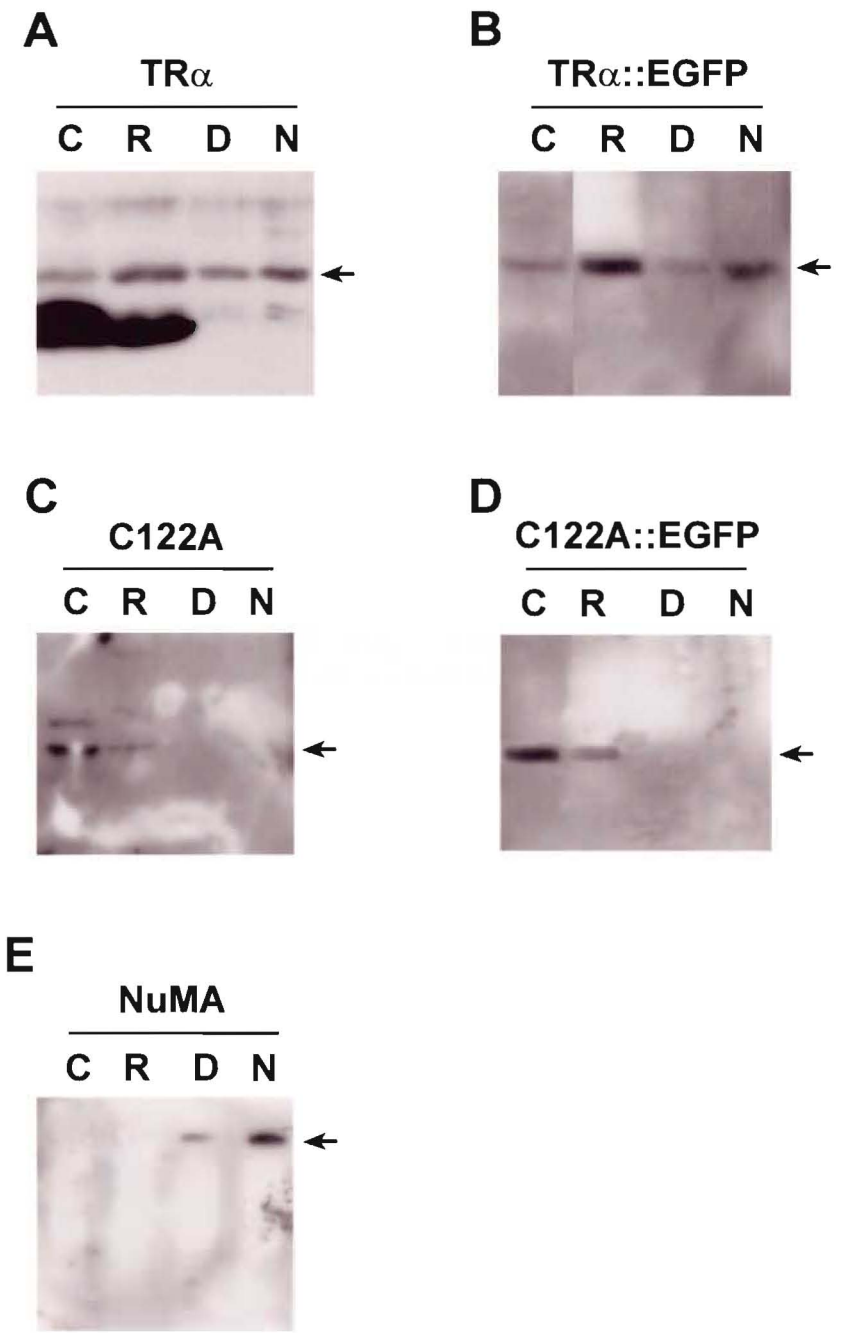
EGFP-tagged receptor distribution was visualised in living cultured mammalian cells. However, the increased size (an extra 27 k Da) and possible artefactual affinities of the EGFP fusion proteins for cellular compartments may have influenced the nuclear distribution of the receptor. Therefore, it was important to determine whether the subnuclear distribution of wild type and tagged receptors differed and, in particular, whether they associated with the nuclear matrix.

Distributions were compared by separation of proteins by PAGE, western transfer, and antibody detection by a well-established protocol (He *et al.,* 1990). The quantity of total cellular protein contained within each of the biochemically-separated fractions was determined prior to separation of samples by SDS-PAGE. As this value determined for each fraction varied, the percentage of TRα::EGFP or C122A::EGFP is expressed relative to the total protein concentration of that fraction. Results of these studies are given in Table 8.

The distributions of TRα and TRα::EGFP isolated by biochemical fractionation differed from that observed for TRα::EGFP in living cells. Whereas no receptor was observed in the cytoplasms of living cells by fluorescent microscopy, the CSK fraction (containing the phospholipids and soluble proteins) contained both forms of receptor (Figure 2.10, A and B). Although 46% of the total cell protein for TRα-transfected cells was contained in the CSK fraction (see Section 2.2.11), only 7% of TRα was detected in this fraction. Likewise, 50% of the total cell protein for TRα::EGFP transfected cells contained only 10% of receptor. Interestingly, the majority of both receptors were detected in the RSB fraction (see Section 2.2.11), which contains the cytoskeleton and polyribosomes. Fifty percent of TRα::EGFP was accounted for in 21% of the total protein isolated, compared with 36% of TRα in 8% of total protein. As expected from a previous study (Perlman *et al.,* 1982), a large amount of both TRα and TRα::EGFP was found associated with the digested chromatin and associated histones (DB), 21% and 10% of 8% total protein, respectively.

Figure 2.10. ***Distribution of TRα, TRα::EGFP, C122A, C122A::EGFP, and EGFP in fractionated NIH 3T3 cells.***

NIH/3T3 cells were transfected with the EGFP fusion vectors. Forty eight hours post-transfection, cells were harvested and rinsed in ice-cold D-PBS. The NIH 3T3 cells were detached and collected by centrifugation, prior to separation of individual cell fractions in various buffers. Fractions were collected in CSK buffer (**C**: to remove phospholipids and soluble proteins whilst leaving the cytoskeleton intact), in RSB-Majik buffer (**R**: to collect the cytoskeleton and polyribosomes), in digestion buffer (**D**: to release the digested chromatin and associated histones), and in digestion buffer without additives (the isolated nuclear matrix intermediate filament (NM-IF, or **N**) pellet). The fractions were separated by PAGE on 12% discontinuous gels containing SDS and analysed after transfer to PVDF membranes. The membranes were probed with anti-TRα antibodies or anti-NuMA antibodies (in the case of untransfected cells). The results are displayed opposite: TRα (**A**); TRα::EGFP (**B**); C122A (**C**); C122A::EGFP (**D**); and untransfected cells (**E**). (n=1)


A
TRα
C R D N
B
TRα::EGFP
C R D N
C
C122A
C R D N
D
C122A::EGFP
C R D N
E
NuMA
C R D N

Lastly, tagged and untagged TRα was found associated with the nuclear matrix-intermediate filament fraction (NM-IF). From 38% total protein, 36% of TRα was contained in this fraction, whereas 20% of TRα::EGFP is contained in 21% of protein. Although this experiment was performed only once, these results give an indication of receptor distribution within each cellular compartment.

C122A distribution was also assessed (both tagged and wild type forms). The 100% cytoplasmic distribution of C122A::EGFP observed in living cells was reflected in the distributions of C122A and C122A::EGFP in fractionated cells (Figure 2.10, C and D). 90% of C122A (in 44% of total cellular protein) was associated with the CSK fraction. Only 5% of C122A was localised in the RSB fraction. Similarly, 95% of C122A (in 30% total protein) was found in the CSK fraction and 10% protein in the RSB fraction. No receptor molecules were detected associated with the chromatin and histones, nor with the NM-IF.

NuMA is a very abundant nuclear matrix phosphoprotein in interphase and relocates to the spindle poles during mitosis (Harboth *et al.,* 1999). NuMA was detected in the chromatin and NM-IF fractions isolated from untransfected cells (DB and NM), as shown in Figure 2.10E. This confirms the integrity of the fractionation procedure for the nuclear matrix. Further fractionation studies would need to be performed with a nuclear protein that is not associated with the nuclear matrix as an extra control.

Table 6 - Summary of receptor distribution studies in *Xenopus* oocytes.

Receptor	*Site of injection*	*Treatment*	*% nuclear protein*	*Student's t-test* [a]
TRα	cytoplasm	physiological conditions	39% ± 3%	reference
TRα	cytoplasm	addition of T_3 hormone	51% ± 3%	p<0.001
TRα	cytoplasm	chilled	40% ± 1%	NS
TRα	cytoplasm	addition of T_3 /chilled	48% ± 3%	P<0.001
TRα	nucleus	physiological conditions	62% ± 7%	Reference
TRα	nucleus	addition of T_3 hormone	85% ± 5%	p<0.001
TRβ	cytoplasm	physiological conditions	39% ± 3%	reference
TRβ	cytoplasm	addition of T_3 hormone	45% ± 1%	P=0.09
TRβ	nucleus	physiological conditions	74% ± 3%	reference
C122A	cytoplasm	physiological conditions	41% ± 2%	reference
C122A	cytoplasm	addition of T_3 hormone	46% ± 1%	P=0.03
C122A	nucleus	physiological conditions	72% ± 6%	reference
v-ErbA	cytoplasm	physiological conditions	21% ± 7%	reference
v-ErbA	cytoplasm	addition of T_3 hormone	20% ± 2%	NS
v-ErbA	cytoplasm	chilled	25% ± 8%	NS
v-ErbA	cytoplasm	addition of T_3 /chilled	20% ± 4%	NS
v-ErbA	nucleus	physiological conditions	87% ± 5%	reference
v-ErbA	nucleus	addition of T_3 hormone	83% ± 5%	NS
L5	cytoplasm	physiological conditions	33% ± 4%	reference
L5	cytoplasm	addition of T_3 hormone	25% ± 4%	P=0.07
L5	nucleus	physiological conditions	68% ± 6%	reference

[a] Student's t-test values are expressed as the probability that the experimental treatments were significantly different at p<0.1. Treatments were compared with reference values obtained in oocytes incubated at physiological conditions, except in the case of combined T_3/0°C treatments where comparisons were made with 0°C incubations. (**NS**: not significant). Sample sizes are given in Figures 2.2, 2.3, 2.5, 2.6, and 2.7.

Table 7 - Summary of EGFP-tagged receptor distribution studies in NIH 3T3 cells.

Receptor	*Sample size (n)*	*Treatment*	*Distribution of protein*	*Transfection efficiency*
TRα	500	physiological conditions	100% nuclear	21% ± 6%
TRα	500	T_3 hormone minus	100% nuclear	20% ± 6%
TRα	500	addition of T_3 hormone (10^{-3}M)	100% nuclear	25% ± 14%
TRα	500	addition of T_3 hormone (10^{-6}M)	100% nuclear	18% ± 8%
TRα	500	addition of T_3 hormone (10^{-9}M)	100% nuclear	20% ± 10%
C122A	297	physiological conditions	100% cytoplasmic	11% ± 5%
C122A	280	T_3 hormone minus	100% cytoplasmic	12% ± 5%
C122A	234	addition of T_3 hormone (10^{-3}M)	100% cytoplasmic	12% ± 7%
EGFP	500	physiological conditions	100% diffused through entire cell	71% ± 13%
EGFP	500	T_3 hormone minus	100% diffused through entire cell	68% ± 13%
EGFP	500	addition of T_3 hormone (10^{-3}M)	100% diffused through entire cell	66% ± 13%

Table 8 - Distribution of receptors in cell fractions.

Nuclear fractions [a]	*TRα* [b]	*TRα::EGFP* [b]	*C122A* [b]	*C122A::EGFP* [b]
CSK	7% (6 µg)	10% (7 µg)	95% (13 µg)	90% (10 µg)
total protein	46%	50%	44%	30%
RSB	36% (1 µg)	50% (3 µg)	5% (3 µg)	10% (12 µg)
total protein	8%	21%	10%	36%
DB	21% (1 µg)	20% (1 µg)	0% (7 µg)	0% (7 µg)
total protein	8%	8%	25%	22%
NM-IF	36% (5 µg)	20% (3 µg)	0% (6 µg)	0% (4 µg)
total protein	38%	21%	21%	12%

[a] CSK: Cytoskeletal buffer (soluble proteins and phospholipids); RSB: RSB Majik buffer (cytoskeleton and polyribosomes); DB: Digestion buffer (chromatin and histones); NM-IF: Nuclear Matrix-Intermediate Filaments

[b] The percentage of receptor localised in each fraction is compared to the amount of total protein in each sample. Micrograms protein loaded per lane for each sample is displayed in brackets.

2.4 Discussion

The distributions of TRα, TRβ, C122A, and v-ErbA in *Xenopus* oocytes, reported in this chapter, differ significantly from those previously reported in cultured cells or those observed in NIH 3T3 cells during the course of this study. Microinjected TR is distributed between the nuclear and cytoplasmic compartments in *Xenopus* oocytes contrary to studies in other cell types which show that TR is constitutively nuclear (Perlman *et al.*, 1982; Puymirat *et al.*, 1989; Macchia *et al.*, 1992; Lee and Mahdavi, 1993). In living NIH 3T3 cells, EGFP-tagged TR was also shown to be localised to the nucleus, whereas C122A was observed only in the cytoplasmic compartment. The possible effects of cellular context are discussed in Section 2.4.1. A reduction in the reported percentage of v-ErbA distributed to the nucleus (Bigler and Eisenman, 1988; Boucher *et al.*, 1988) was also observed in *Xenopus* oocytes. Heat shock protein 90 was shown to associate with v-ErbA, which would account for the sequestering of v-ErbA in the oocyte cytoplasm (Section 2.4.5).

DNA binding was shown not to be essential for nuclear retention of TR, as DNA-binding mutant C122A was localised to the oocyte nucleus to the same extent as TRα or TRβ. This suggests that TR may associate with other nuclear binding sites apart from TREs (Section 2.4.2). In addition to associating with the chromatin and histone fraction, TR was seen to associate with the nuclear matrix fraction of biochemically fractionated 3T3 cells. C122A was not found in either of the nuclear fractions, confirming the observations made in living cells. Incubation of microinjected oocytes in T_3 resulted in a shift of TRα, TRβ, and C122A, but not v-ErbA, to the nuclear compartment. No equivalent shift of EGFP-tagged TRα or C122A was observed in NIH 3T3 cells. The implications of these observations are discussed in Section 2.4.3.

2.4.1 Localisation of receptor is dependent on cell type

The subcellular distributions of TRα and C122A vary greatly between *Xenopus* oocytes and cultured NIH 3T3 cells. Whilst both TRα and C122A have approximately 40%

of receptor localised to the nuclei of oocytes, in NIH 3T3 cells TRα is constitutively nuclear and C122A is excluded from the nuclei.

The presence of cytoplasmically injected TRα in *Xenopus* oocytes was not shown to be due to a saturation effect. No difference in TRα distribution was observed for three concentrations of microinjected protein (50, 100, or 200 picograms per 50 nanolitre injection). Cytoplasmically introduced TRα reached a steady state at approximately five hours after microinjection and this steady state was monitored throughout a twelve hour period. In addition, when the same amount of protein was introduced to the nuclear compartment, a greater proportion was retained in the nucleus. This observation suggests that if cytoplasmically introduced receptor had entered the nucleus to a greater extent than that observed, a greater proportion of receptor would have been retained in the nucleus. As this is not the case, it suggests that nuclear retention sites are available for further binding and that nuclear accumulation was not saturated. Therefore, the difference in TRα distribution between cell types may be attributed to the cellular context; that is, the presence or absence of factors associated with transport mechanisms or anchoring proteins, as well as accessibility to these factors.

A further way of testing for saturation of nuclear binding sites in *Xenopus* oocytes would be to introduce extra binding sites into the cell nucleus and then assay for a shift in TR distribution. A range of TREs incorporated into vectors is available for introduction by microinjection. A shift in receptor distribution could be assayed for by the standard protocols described in Section 2.2.4. In an attempt to investigate this aspect and in conjunction with nuclear retention site studies, additional sites were provided by way of nuclear preinjection of a synthetic TRE linked to a mouse mammary tumour virus (ΔMTV) LTR-CAT fusion gene (TREp: Nelson *et al.,* 1993) during the course of the investigations described in this thesis. However, although cycloheximide was added to the incubation medium to prevent synthesis of proteins from unincorporated [^{35}S]-methionine, present in the [^{35}S]-TRα sample, expression of CAT produced enough background to obscure the [^{35}S]-TRα band on the autoradiograms. No TRE constructs, excluding CAT fusions, were available in the laboratory for testing, thus these analyses were not pursued further.

Several explanations for the observed variation in TR distribution are proposed. Firstly, cytoplasmic factors responsible for TR import may be absent in oocytes, or present at reduced levels. Secondly, retention sites in the nuclei of NIH 3T3 cells may be more abundant than those available in *Xenopus* oocytes (discussed in Section 2.4.2). Alternatively, T_3 binding may not be necessary for nuclear import of TR in cultured cells, whilst it may be necessary for release from an associated cytoplasmic factor in oocytes. The effects of hormone binding on nuclear localisation will be discussed in Section 2.4.3. It has been observed that several proteins redistribute between the nuclear and cytoplasmic compartments at precise stages in embryonic development (reviewed in Nigg, 1990). Moreover, transport rates are faster and the functional sizes of NPCs are significantly larger in proliferating cells than in quiescent cells (Feldherr and Akin, 1994). These observations support the idea that differences in cellular context may be implicated in receptor distribution, particularly when comparing oocytes with somatic cells.

Members of the NHR superfamily have been shown to associate with complexes containing hsp90 and associated factors. GR, AR, and MR are sequestered in the cytoplasm by hsp90 and upon hormone binding are released from this association prior to transport to the nucleus (reviewed in Feldherr and Akin, 1994; reviewed in Brasch and Ochs, 1995; Dittmar *et al.,* 1996). TRα has not been isolated complexed with hsp90; however, it is possible that TRα may form an association with another type of cytoplasmic anchor. Cytoplasmic anchors regulate nucleocytoplasmic transport, and therefore cell signalling, by two general mechanisms: anchoring and release of the protein, or masking and unmasking the NLS. For example, endoplasmic reticulum membranes and cytoplasmic membranous organelles are directly involved in anchoring sterol-regulatory element binding proteins and inactive phosphokinase A (PKA) holoenzyme (Wang *et al.,* 1994; Nigg, 1990; respectively). Conversely, IκB masks the NLS of NFκB/Rel, inhibiting import (Baeuerle and Henkel, 1994). Phosphorylation and proteolysis of IκB inactivates the cytoplasmic anchor, allowing NFκB to enter the nucleus and bind DNA.

The presence of a cytoplasmic anchor may be supported in part by the results of experiments carried out by Dr L. A. Allison on my behalf in her new laboratory (College of William and Mary, Williamsburg, VA, USA). It was observed during the course of this study

that TRα molecules in the cytoplasmic and nuclear fractions, extracted from microinjected oocytes, migrated differently during gel electrophoresis. Nuclear TRα migrates with a slower mobility than cytoplasmic TRα (Figure 2.2). However, when the nuclear fraction is treated by a dephosphorylation reagent (calf intestinal alkaline phosphatase), mobility of TRα is identical to that of cytoplasmic TRα (L. A. Allison; personal communication). To summarise, these pilot studies indicate that nuclear TRα is phosphorylated. This suggests that release from an anchor protein requires phosphorylation at one or a combination of the four possible phosphorylation sites detected during a search for protein motifs carried out through PROSITE (http://expasy.hcuge.ch/sprot/prosite.html).

The percentage of v-ErbA present in the nucleus of oocytes determined during the course of this study is less than the 30-40% reported in the literature for cultured cells nuclei (Privalsky 1991; 1992). This variation may be due to hsp90 binding, and will be discussed in Section 2.4.5.

The difference observed in TRα distribution between cell types could be attributed to the use of EGFP-tagged receptors in NIH 3T3 cells rather than due to differences in cell type. Addition of the 238 amino acid EGFP tag to TR and C122A for the cultured cell studies accounts for a sizeable increase in receptor size (of 27 kDa). In order to test whether import was affected by the EGFP fusion, control experiments would need to be performed with untagged receptor molecules that could be subsequently detected with specific antibodies, prior to detection by fluoroscein-labelled secondary antibodies and analysis by fluorescence microscopy. Although these studies were attempted, non-specific background masked any TR specific detection, and time constraints did not allow optimisation. Alternatively, EGFP constructs could be utilised in *Xenopus* oocytes. However, due to the pigmentation of the cell and auto-fluorescence of the yolk pigments, it would be necessary to detect receptor localisation by manual dissection of oocytes and subsequent immunoblotting. Interestingly, addition of a tag 3 kDa larger than GFP, GST, to TR produces a fusion protein that has been shown to be similarly distributed to TR in Dr L. A. Allison's laboratory (College of William and Mary; personal communication).

Addition of a GFP tag may alter other properties of TRα or C122A, apart from size. In particular, DNA binding, hormone binding, and transactivation of gene expression need to be assessed, in order to determine whether altered distribution or transport characteristics could be attributable to factors other than size of receptor molecules. Data obtained in Dr L. A. Allison's laboratory (College of William and Mary) by electrophoretic mobility shift assays (EMSA) show that EGFP-tagged TRα binds a TRE, although there is no hormone binding data at present (personal communication).

The substantially increased receptor size raises the likelihood that nuclear import is affected by the increased molecular weight (discussed in Section 2.4.4). In addition, whilst isolating the NM-IF to investigate whether TRα and C122A associate with nuclear matrix, it was observed that a fraction of both TRα and C122A, with and without GFP tags, was isolated with the soluble proteins of the cytoplasm, the polyribosomes, and the cytoskeleton. Therefore it is possible that a population of receptor is present in the cytoplasm of cultured cells that is not visible by fluorescence microscopy, or that it represents newly synthesised protein just released from the ribosomes. Alternatively, the fractionation procedure may not have produced very clean fractions. Verification of receptor localisation with confocal microscopy and laser imaging would be appropriate, as a three dimensional image through the cell is obtained at a greater resolution than is possible with 35 mm photography through a fluorescent microscope.

2.4.2 Localisation of TR is not solely dependent upon DNA binding in Xenopus oocytes

Nuclear accumulation of proteins can be separated roughly into two general classes (reviewed in Nigg, 1990), although these are not mutually exclusive. The first contains those proteins that are localised to the nucleus by a signal-directed mechanism and targeted to sites of action. Proteins in the second class enter the cell nucleus by passive diffusion and are retained in the nucleus by associations with nuclear proteins or complexes, through selective binding. Due to the constitutive DNA localisation of TR reported in somatic cells (Perlman *et al.,* 1982; Kumara-Siri *et al.,* 1986; Horowitz *et al.,* 1989; Puymirat *et al.,* 1989; Macchia *et al.,* 1992; Lee and Mahdavi, 1993), the mechanism of nuclear accumulation of TR has not

been previously investigated. Therefore, nuclear accumulation of TR in *Xenopus* oocytes could be due to signal-directed import and association with DNA or it may be due to passive diffusion and retention by association with nuclear factors or specific retention sites. In *Xenopus* oocytes, no significant difference is observed between the distributions of wild type TR isoforms and DNA-binding mutant C122A. This suggests that nuclear accumulation may be due, in part, to retention by association of TR with binding sites, other than DNA, in the cell nucleus. In light of these observations for TR, it is interesting to note that nuclear localisation and tight binding of GR to nuclei is affected by DNA binding (Sackey *et al.*, 1996). Other sites for nuclear retention could include components of the nuclear matrix (discussed in Section 2.4.4), as has been reported for other members of the NHR superfamily (described in Section 2.1.3).

C122A is excluded from the nucleus whereas TRα is intranuclear in cultured NIH 3T3 cells. If nuclear import of TRα is by a facilitated mechanism in NIH 3T3 cells, then disruption of an NLS in the DBD, by the mutation at position 122, might prevent import. However it seems unlikely that such a disruption would occur, although it cannot be eliminated, as the only NLS identified in wild type TRα to date is found further towards the carboxyl terminal (Dang and Lee, 1989; Lee and Mahdavi, 1993), as is the case for TRβ (Zhu et al., 1998). The localisation of C122A to *Xenopus* oocyte nuclei and its exclusion from NIH 3T3 nuclei indicates again that cellular context is important in receptor distribution. Different cellular contexts have been shown to be associated with varying GR and MR distributions. Immunocytochemical studies showed that, independent of ligand binding, MR was nuclear in rabbit kidney cells (Farman *et al*, 1991), and that unliganded GR was nuclear in chinese hamster ovary (CHO) cells (Sanchez *et al.*, 1990). In contrast, unliganded GR and MR remained cytoplasmic in rat neurons (Fuxe *et al.*, 1985; Wikström *et al.*, 1987; Rossini and Malaguti, 1994; Akner *et al.*, 1995) and over-expressing Sf9 insect cells and mouse macrophages (Robertson *et al.*, 1993). There are several possible reasons for this variation. Firstly, unspecified cytoplasmic factors responsible for C122A transport may be absent in NIH 3T3 cells. This possibility seems unlikely as C122A is a synthetic mutant, varying in only one amino acid residue, and it would be expected that transport occurs through the same factors as those responsible for wild type TRβ transport. Secondly, the binding specificity of these transport factors may vary between cell types; such that, whilst C122A is recognised

and transport is aided in oocytes, the factors in cultured mammalian cells may not recognise the mutated TRβ receptor. Thirdly, anchoring factors that associate with C122A may be present in *Xenopus* oocytes and to a greater degree in NIH 3T3 cells. However, if this is the sole reason for the 100% cytoplasmic localisation of C122A in NIH 3T3 cells, then it is unlikely that T_3 would play a role in anchor release as suggested in oocytes, due to a lack of observable effect when NIH 3T3 cells were incubated in 10^{-3} M hormone (discussed further in Section 2.4.3).

Further aspects of the cellular environment to consider are the nuclear retention sites, not including DNA, as C122A does not bind DNA. Yamamoto and Alberts proposed a two-site DNA model for NHR action (reviewed in Nigg, 1990). It was proposed that NHRs associate with a large number of low affinity sites in the nucleus, and a smaller number of high affinity sites. In addition, specific binding to these higher affinity sites would be masked by the large number of low affinity sites. This model could be applied to TR, suggesting that, while DNA binding is observed, TR may also associate with a number of other sites with varying degrees of affinity. Modulation of the receptor by hormone binding may in turn increase the number of sites for potential binding, or may result in a rearrangement of TR distribution amongst these sites due to altered affinities (refer Section 2.4.3). The conformation of nuclear binding sites or the accessibility of these sites may also be more available in oocytes, allowing a closer association of receptor with these retention sites. These sites may be present in a less accessible conformation in cultured mammalian cells, if they are in fact present. If nuclear localisation is dependent upon binding to retention sites, then lack of association with subnuclear compartments could conceivably lead to egress of C122A from the nucleus. However, it appears unlikely that C122A reaches the nuclear compartment in NIH 3T3 cells in any great concentration as no receptor was detected in the nuclear fractions during the isolation of NM-IFs (refer Section 2.4.4).

In order to test whether C122A is not retained in the nucleus, or whether it is simply not imported in cultured NIH 3T3 cells, specialised microinjection equipment for use with cultured cell lines would need to be utilised. C122A could be introduced to the nuclei of NIH 3T3 and other suitable cell lines by microinjection. Distribution of receptor could be determined with the use of EGFP tags or through antibody detection as discussed in the

previous section. Export of receptor from the cell nucleus would be identified, supporting the proposal that there are insufficient, if any, binding sites available rather than a lack of specific transport factors.

Lastly, factors may be present in the oocyte that aid in association of receptor molecules with retention sites. Presumably, these factors would be absent or present in reduced levels in NIH 3T3 cells, or would be unable to recognise the mutated C122A whilst retaining the ability to recognise TRα.

2.4.3 Influence of T_3 hormone binding on receptor localisation

The incubation of *Xenopus* oocytes in T_3 resulted in a significant shift of TRα, TRβ, and C122A to the nuclear compartment in both nuclear and cytoplasmically injected oocytes. However, v-ErbA distribution remained unaffected by addition of hormone. This observation was expected as mutations in the v-ErbA HBD render it unable to bind T_3 at physiological levels.

The shift of TRα, TRβ, and C122A induced by T_3 suggests several possibilities for the role T_3 plays in receptor distribution. Firstly, it seems likely that hormone binding allows receptor molecules to associate with additional nuclear binding sites. The increased affinity for these sites may be due to the alteration in receptor conformation induced by the hormone binding (Toney *et al.*, 1993), making the sites more accessible and therefore allowing a closer association of receptor with these retention sites. In support of this idea, it was recently reported that T_3 induced an enhanced rate of translocation of EGFP-tagged TRβ to the nuclei of CV1 cells (Zhu *et al.*, 1998). Mutation of the Lys-Arg residues (184-185) of this TRβ-GFP chimera to Ala-Ala blocked this hormone induction. An alternative role for hormone could be in the release of TR from a cytoplasmic anchoring protein, as is the case for GR, AR, and MR (Yang and DeFranco, 1994).

Distribution of TRα::EGFP and C122A::EGFP is not influenced by addition of hormone to cultured NIH 3T3 cells. Therefore, whilst T_3 binding may not be necessary for

nuclear import of TR in cultured cells, it appears to play a role in nuclear import of TRα, TRβ, and C122A in *Xenopus* oocytes. Further analysis of TRα and C122A receptors in cultured cells with confocal microscopy might indicate any fine alterations in distribution within the cellular compartment (nuclear or cytoplasmic, respectively) of hormone-treated cells. Alternatively, indirect analysis of fixed cells with antibodies could be performed although this would be less sensitive. Thirdly, as discussed in the previous section, anchoring factors that associate with C122A may be present in *Xenopus* oocytes and to a greater degree in NIH 3T3 cells.

2.4.4 Subnuclear distribution of receptors in cultured mammalian cells

A nuclear accumulation model has been proposed, based on binding of proteins to `quasifunctional binding sites', allowing gene regulation at the level of organisation of the cell nucleus (reviewed in Bonner, 1978). This model proposed that the measured binding of a protein in a cell nucleus may not be its ultimate functional binding, but rather reflects binding to low affinity sites prior to association with control sites. Alternative sites for nuclear retention, apart from DNA binding sites, could include components of the nuclear matrix, as has been reported for other members of the NHR superfamily. Association of ER and GR with the nuclear matrix has been detected (Alexander *et al.*, 1987; van Steensel *et al.*, 1995). Receptors that bound T_3 were isolated from the nuclear matrix of cultured GC cells by photoaffinity labelling of receptors by [^{125}I]-T_3 (Kumara-Siri *et al.*, 1986). In the investigations described in this thesis, subpopulations of both TRα and TRα::EGFP were isolated from the NM-IF fractions of TRα and TRα::EGFP transfected cells. Therefore, these results support an association of TRα with the nuclear matrix of NIH 3T3 cells.

No cytoplasmic population of EGFP-tagged receptor was observed in living cells although TRα molecules (wild type and EGFP-tagged) were distributed throughout fractionated transfected cells when analysed by immunoblotting. Up to 60% of receptor was observed associated with the cytoskeleton, polyribosomes and soluble cytoplasmic proteins. However, it must be remembered that the biochemical fractionation experiment was performed only once and did not have internal controls for all the fractionation steps. No

cycloheximide was added to the growth medium to arrest protein synthesis prior to fractionation of the cells, as in the case of living cell studies. Therefore, newly synthesised protein would be present within the cytoplasmic fractions that had not yet been localised. Further, as western analysis is more quantitative than observation of living cells by fluorescent microscopy, it is feasible that a small diffuse cytoplasmic pool of receptor in living cells might remain undetected. Indeed, support for the existence of a cytoplasmic form of receptor was provided recently when a TRβ-GFP chimera was shown to be present in both the nuclear and cytoplasmic compartments by confocal microscopy (Zhu *et al.,* 1998).

Similar concentrations of TRα and TRα::EGFP were present in the nuclear fractions of biochemically fractionated cells when compared with the amount of total protein contained within each fraction. Although replicates of this experiment were not performed, these initial studies suggest that it is unlikely that localisation of TRα to the nuclei of NIH 3T3 cells is affected by the increased molecular size of the EGFP-tagged receptor.

The distributions of untagged and EGFP-tagged C122A detected by antibody were the same and their distribution was consistent with that of C122A::EGFP in living cells (Section 2.3.3). Receptor was only isolated from the cytoplasmic fractions of the cells, as was the case for EGFP-tagged C122A in living cells (Section 2.4.2). No C122A was associated with the chromatin, histones, or the NM-IF fraction. This finding suggests a role for DNA binding in receptor localisation. However, it must be cautioned that disruption of the DBD may also cause disruption of other regions necessary for correct cellular localisation (discussed further in Section 3.4.1 with respect to NLSs).

2.4.5 v-ErbA associates with hsp90

Rabbit reticulocyte lysate has been used for the coupled *in vitro* transcription/translation of a variety of proteins (Murdoch and Allison, 1996; Nagl *et al.,* 1997). This reticulocyte lysate also contains a multiprotein system that complexes NHRs with endogenous hsp90 (Dittmar *et al.,* 1996). As discussed in Section 2.1.4, association with hsp90 renders the NHR unable to bind DNA. Due to this association, the NHR HBD is

transformed to an open hormone-binding conformation. Only after hormone binding and transformation of the NHR to an open DNA binding conformation, may the receptor be released from the cytoplasmic anchoring complex. It has been proposed that association of NHR with hsp90, rendering receptor unable to bind DNA, directly affects the cellular localisation of the receptor in a hormone-free environment (Dalman *et al.,* 1990). Whilst v-ErbA has been shown to associate with hsp90, TR has not (Section 2.1.4).

A difference between the distributions of TR (including both isoforms and the TRβ mutant, C122A) and v-ErbA is observed in *Xenopus* oocytes (discussed in Section 2.4.1). Whilst approximately 40% of TR is found in the nuclei of *Xenopus* oocytes, only 20% of v-ErbA is localised to the nucleus. As rabbit reticulocyte lysate, in which receptors were translated *in vitro*, contains hsp90 (Dittmar *et al.,* 1996), the ability of v-ErbA to complex with hsp90 may account for the difference in distribution between TR and v-ErbA in the oocyte system. As v-ErbA can complex with predominantly cytoplasmic hsp90, the receptor could be anchored in immobile complexes in the cytoplasmic compartment and would therefore not be free to enter the nucleus through the NPCs until dissociation of hsp90 from receptor occurred.

Association of v-ErbA with hsp90 in the *in vitro* translation mixture was confirmed. Although the presence of this complex was verified, it was not determined whether hsp90 was responsible for anchoring v-ErbA in the oocyte cytoplasm. In order to pursue this line of reasoning, an expression vector for v-ErbA would need to be introduced into the oocyte nucleus for the endogenous production of receptor as opposed to the introduction of exogenous protein formed in the presence of hsp90. Isolation of the receptor from oocyte homogenates and analysis according to the protocol described in Section 2.2.6 for the translation mixture, would allow the effects of hsp90 present in rabbit reticulocyte lysate to be assessed. Although the presence of hsp90 in *Xenopus* oocytes has been suggested (Uzawa *et al.,* 1995; Heikkila *et al.,* 1997), the concentration of a pool of hsp90 is unlikely to be consistent with that contained within the reticulocyte lysate mixture. Therefore, variation in endogenous-produced receptor localisation from that of introduced receptor might be indicative of hsp90 interactions and subsequent cytoplasmic retention.

Chapter 3

Nucleocytoplasmic Transport of the Thyroid Hormone Receptor and Variants

3.1 Introduction

TR and its variants are not constitutively localised to the nuclei of *Xenopus* oocytes, as presented in Chapter Two. This novel finding suggests that nuclear localisation of receptor may not be due entirely to DNA binding (supported by the nuclear accumulation of DNA-binding mutant C122A), and therefore, a comparison of the mechanisms of transport, into and out of the oocyte nucleus, of TR and variants was necessary. Differences in nucleocytoplasmic transport mechanisms could lead to variation in receptor availability for nuclear retention or export. A lack in resolution obtained through standard immunocytochemical staining techniques may mean that fine-tuned regulation of TR nuclear localisation has been missed in cultured mammalian cells. Therefore, oocytes provide a good system in which to manipulate the factors involved in trafficking of the receptor due to the difference, from the accepted dogma, in distribution, and the possibility of studying both import and export in quantitative terms.

This chapter addresses whether TR and variants are imported into or exported from the nuclei of *Xenopus* oocytes by a facilitated mechanism or by passive diffusion. Whilst TRα, TRβ, and C122A fall within the upper size limits for passive diffusion through the NPC (46.8 kDa, 52 kDa, and 52 kDa, respectively), v-ErbA (with the viral *gag* protein fusion) exceeds the limit (75 kDa). Comparison of the nucleocytoplasmic transport of DNA-binding mutant C122A with that of wild-type receptor was performed to assess whether the integrity of DBDs alters the mechanism of transport. Results obtained in oocytes are compared with those obtained by tagging TRα and C122A with GFP in cultured NIH 3T3 mammalian cells.

3.1.1 Nucleocytoplasmic transport

Modification of the classical model for steroid hormone action has occurred due to elucidation of a diversity of mechanisms regulating nucleocytoplasmic transport of different members of the NHR superfamily that are in opposition to the model. It was proposed in the classical model of steroid hormone action that cytoplasmic pools of receptor were translocated to the nucleus after hormone binding, and that liganded receptor is associated with HREs prior to activation or repression of gene transcription (reviewed in Nigg, 1990). However, localisation of receptors does not appear to be a static, unidirectional process, but instead seems to reflect shuttling of receptor molecules between the nucleus and cytoplasm. This dynamic situation, or nucleocytoplasmic shuttling, involves the transport of receptor molecules between the nuclear and cytoplasmic compartments. Identification of the mechanisms that control receptor shuttling has been the focus of several studies to date (presented in Table 9).

3.1.2 Nucleocytoplasmic transport of nuclear hormone receptors

The receptor for progesterone has been shown to shuttle between the nuclear and cytoplasmic compartments (Guiochon-Mantel *et al.,* 1991). Initially it was observed that cytoplasmic and nuclear PR monomers interacted (Guiochon-Mantel *et al.,* 1989). This observation was supported by experimentation with a series of deletion mutants and interspecies heterokaryon formation (Guiochon-Mantel *et al.,* 1991; 1994). Whilst import of PR requires energy, export is energy independent. However, PR export is not by diffusion, but like import it is NLS-directed (Guiochon-Mantel *et al.,* 1994).

Import of GR is via a facilitated mechanism (Yang and DeFranco, 1994). This process is energy and temperature dependent and takes place after hormone binding. In addition, export occurs upon hormone dissociation. Nucleocytoplasmic shuttling of GR has also been demonstrated in heterokaryon experiments similar to those carried out for PR (Madan and DeFranco, 1993). Nucleocytoplasmic transport of AR and MR also appear to take place by a similar process to GR, with similar requirements (Yang and DeFranco, 1994). Although VDR

is predominantly nuclear, a portion of receptor is found in the cytoplasm, suggesting nucleocytoplasmic shuttling occurs (Bidwell *et al.,* 1994; Barsony *et al.,* 1997).

Table 9 - Transport of nuclear hormone receptors.

NHR	*Experimental*	*Requirements*	*Observations*	*References*
GR	Heterokaryons	Hormone binding	Shuttling	Madan and DeFranco, 1993
	In vitro and *in vivo* (dissociation from hsp90)	Hormone binding ATP/temperature dependent	Import	Yang and DeFranco, 1994
	In vitro and *in vivo* (dissociation from hsp90)	Dissociation of hormone	Export (release from chromatin)	Yang and DeFranco, 1994
	In vitro export in permeabilized cells	Dissociation of hormone ATP independent	Chromatin release to low affinity nuclear compartment (export staging area)	Yang *et al.,* 1997
AR	*In vitro* and *in vivo* (dissociation from hsp90)	Hormone binding ATP/temperature dependent	Import	Yang and DeFranco, 1994
	In vitro and *in vivo* (dissociation from hsp90)	Dissociation of hormone	Export (release from chromatin)	Yang and DeFranco, 1994
MR	*In vitro* and *in vivo* (dissociation from hsp90)	Hormone binding ATP/temperature dependent	Import	Yang and DeFranco, 1994
	In vitro and *in vivo* (dissociation from hsp90)	Dissociation of hormone	Export (release from chromatin)	Yang and DeFranco, 1994
ER	Anti-oestrogen	Hormone binding	Shuttling	Dauvois *et al.,* 1993
PR	Deletion mutants (qualitative only)	Not defined	Shuttling of PR monomers	Guiochon-Mantel *et al.,* 1989
	Deletion mutants and ATP depletion	Energy independent	Export of PR	Guiochon-Mantel *et al.,* 1991
PR	Heterokaryons	Energy independent export but energy dependent import	Shuttling (identified after 12-18 hours)	Guiochon-Mantel *et al.,* 1991
	NLS mutants β-galactosidase fusions	NLS required (import and export) Energy dependent (import only)	Shuttling	Guiochon-Mantel *et al.,* 1994

Although TR is widely accepted as being constitutively nuclear in cultured mammalian cells (Perlman *et al.,* 1982; Kumara-Siri *et al.,* 1986; Horowitz *et al.,* 1989;

Puymirat *et al.,* 1989; Macchia *et al.,* 1992; Lee and Mahdavi, 1993), a significant amount of chicken TR is found in the cytoplasm when transiently expressed at high levels in a rat cell line (Horowitz *et al.,* 1989). This suggests that localisation of receptor may be dependent on the availability of saturable receptor sites in the nucleus. Further, the results presented in Section 2.3.2 of this thesis clearly demonstrate that approximately 60% of cytoplasmically-introduced receptor molecules exist cytoplasmically in *Xenopus* oocytes, and that this is not simply due to a saturation effect. Therefore, a case is made to examine the nucleocytoplasmic transport mechanisms involved in nuclear localisation of TR.

3.1.3 Differentiating facilitated transport from diffusion

Protein transport cannot be categorised into facilitated or passive processes simply upon size. Several molecules that are smaller than the 40-60 kDa size limit for diffusion are actively transported. For example, histone H1 (21 kDa) nuclear import was shown to be inhibited by chilling, energy depletion, WGA, and addition of non-hydrolysable GTP analogues in digitonin permeabilised cell assays (Breeuwer and Goldfarb, 1990; Kurz *et al.,* 1997). Calmodulin (16.8 kDa) import is inhibited by WGA and chilling, but not energy depletion, when assayed by tissue culture cell microinjection and permeabilized cell import. This suggests that calmodulin import occurs by a facilitated diffusion process and not an active mechanism (Pruschy *et al.,* 1994). Although histone H1 import appears to take place through an active, receptor-mediated mechanism, no putative NLS has been identified. In the case of the 41 kDa catalytic subunit of cAMP-dependent protein kinase, the protein has lysine and arginine residues that could comprise an NLS. However, Harootunian and colleagues (1993) concluded that diffusion was sufficient to explain most aspects of the subunit's transport.

Interestingly, as noted earlier in this section, WGA and chilling, but not energy depletion inhibit nuclear import of calmodulin; thus, facilitated pathways may be further distinguished through a requirement for energy. When a combination of these three assays is used, passive diffusion can be characterised as being distinct from facilitated and active transport mechanisms.

As described in Section 1.2.3, facilitated transport occurs via a two step process: docking of cargo protein, complexed with transport factors (such as importin α/β or transportin), at the NPC followed by translocation through the NPC. Inhibition of docking and translocation can be applied to determine the general mechanism for protein transport; that is, to discriminate between passive diffusion and signal-directed facilitated processes. The requirements for protein transport, and methods of inhibition are discussed in the following sections (Sections 3.1.4 to 3.1.7). In addition to assays assessing energy requirement, temperature dependence, and WGA inhibition by binding to the nucleoporins of the NPC, competition assays with ribosomal protein L5 and histone H1 were also used to assess alterations in transport kinetics.

3.1.4 Energy requirements for transport

Controlled protein transport (facilitated transport) through the NPC may occur by an energy dependent process as discussed in Chapter One (Section 1.2.2). Therefore, a requirement for energy can be used to distinguish facilitated transport from passive diffusion (Newmeyer and Forbes, 1988; Richardson *et al.,* 1988; Akey and Goldfarb, 1989). The two stages of transport can be separated by depletion of energy, or by chilling of the transport system to 4°C (Newmeyer and Forbes, 1988; Richardson *et al.,* 1988). Transport is arrested at the docking stage and may be continued by addition of energy or reversion to a physiological temperature.

Although it appears that the requirement for ATP is indirect, and rather that GTP hydrolysis mediated by GTPase Ran and accessory proteins is necessary (as discussed in Section 1.2.3), depletion of ATP (which also depletes GTP) has been used to distinguish between passive and facilitated mechanisms. ATP can be depleted by the addition of hydrolysing agents to test whether transport occurs after ATP depletion. Such agents have been used to hydrolyse ATP and thereby characterise energy requirements during transport of proteins and ribonucleoprotein particles in several systems, including *Xenopus* oocytes (Bataillé *et al.,* 1990) and cells in tissue culture. There is a stringent requirement for energy during the transport of ribosomal subunits (Bataillé *et al.,* 1990), mRNA (Dargemont and

Kühn, 1992), and viral RNPs (Kemler *et al.,* 1994), histone H1 import in PtK1 cells (Breeuwer and Goldfarb, 1990; Kurz *et al.,* 1997), nucleocytoplasmic transport of GR, AR and MR (Yang and DeFranco, 1994), and import of PR (Guiochon-Mantel *et al.,* 1994). In addition to the use of hydrolysing agents, the matrix protein of vesicular stomatitis virus (VSV M protein), introduced into oocytes, was shown to inhibit Ran GTPase-dependent transport of small nuclear RNPs (Her *et al.,* 1997).

3.1.5 Temperature dependence

A further criterion for establishing the mechanism of transport is to assess temperature dependence of the import or export process. At low temperatures, 0°C to 4°C, facilitated and active transport are inhibited whilst passive diffusion continues, although reduced to approximately 90% of the normal rate (Breeuwer and Goldfarb, 1990; Kambach and Mattaj, 1992). Lowering the incubation temperature of the cellular environment during transport decreases the rates of enzyme-mediated processes.

Experimentally, chilling has been shown to inhibit facilitated nuclear import of a variety of karyophilic proteins (reviewed in Wagner *et al.,* 1990; Murdoch and Allison, 1996), including nucleoplasmin in tissue culture cells (Richardson *et al.,* 1988), histone H1 (Breeuwer and Goldfarb. 1990; Kurz *et al.,* 1997), and calmodulin (Pruschy *et al.,* 1994). Nuclear export of ribosomal subunits (Bataillé *et al.,* 1990), signal recognition particle RNA (He *et al.,* 1994), and U snRNAs (Jarmolowski *et al.,* 1994) are also temperature dependent processes. As discussed in Section 3.1.2, nucleocytoplasmic transport of some members of the NHR superfamily is inhibited by low temperature incubations. In contrast, small proteins lacking NLSs diffuse through the nuclear pores regardless of temperature (Breeuwer and Goldfarb. 1990).

3.1.6 Competition assays

Another approach to block nucleocytoplasmic import is to use a competitive inhibitor of facilitated or active protein import. Experiments performed *in situ* suggest that whilst some U snRNPs may be imported by alternative pathways, the majority of karyophilic proteins are imported by a shared pathway and therefore interact with the same import receptor complexes (Michaud and Goldfarb, 1992; 1993). Therefore, as active and facilitated transport of proteins are saturable processes, they are sensitive to competition by other substrates that follow the same pathway, such as histone H1 (Breeuwer and Goldfarb, 1990; Harootunian *et al.,* 1993; Kurz *et al.,* 1997). The nuclear import of rhodamine (rd)-nucleoplasmin was somewhat inhibited by excess histone H1 (810 μM), and fluoroscein-labelled histone H1 (24 μM) by an equivalent concentration (24 μM) of unlabelled histone H1 (Breeuwer and Goldfarb, 1990).

3.1.7 Inhibition of transport by wheat germ agglutinin

The cytosolic factors required for docking, discussed in Section 1.2.3, are distinct from those necessary for translocation (reviewed in Davis, 1995). The second stage of transport, or translocation, can be inhibited by a variety of agents; for example, addition of wheat germ agglutinin (WGA) or anti-FG nucleoporin repeat antibodies (nucleoporins containing highly repetitive repeats of the amino acids phenylalanine (F) and glycine (G): reviewed in Davis, 1995), Ran GTPase-inhibiting VSV M protein (Her *et al.,* 1997), dominant-negative mutants of importin β that bind to the NPC but not to Ran (Kutay *et al.,* 1997), and non-hydrolysable analogues of GTP (Breeuwer and Goldfarb, 1990; Kurz *et al.,* 1997).

WGA is a plant-derived lectin that was originally shown to inhibit the ATP-dependent release of ribonucleoproteins in isolated rat nuclei (Baglia and Maul, 1983) and has now been used extensively in transport studies. Lectins are substances that bind to *N*-acetylglucosamine residues (GlcNAc) on glycoproteins. Nucleoporins, which are essential components of the NPC, with respect to structure and function, are glycoproteins with GlcNAc joined by O-linkages to the polypeptide backbone (Holt *et al.,* 1987). Various lectins have been tested

for their ability to bind to the NPC (Baglia and Maul, 1983; Finlay *et al.,* 1987; Gerace, 1992). Fluorescently labelled WGA and another lectin, concanavalin A (Con A) were shown to bind to the periphery of nuclei in a punctate staining pattern, consistent with the lectins binding to nuclear pores (Finlay *et al.,* 1987; Yoneda *et al.,* 1987). However, only WGA inhibited the nuclear import of nucleoplasmin. WGA has a high affinity for clusters of GlcNAc residues. Further to the studies with fluorescently-labelled WGA, it has been demonstrated through the use of gold-conjugated WGA (Akey and Goldfarb, 1989; Panté and Aebi, 1994) and ferritin-labelled WGA (Finlay *et al.,* 1987; Allen, 1990) that WGA binds to the central region of the NPC. In the latter study, WGA conjugated to ferritin was observed bound to fibrous material at the pore margins in cultured mammalian cells.

Subsequently, inhibition of nucleocytoplasmic transport by WGA has been shown to occur *in vivo* in both *Xenopus* oocytes (Dabauvalle *et al.,* 1988) and in cultured cells (Yoneda *et al.,* 1987; Wolff *et al.,* 1988). Although WGA blocks the uptake of a variety of karyophilic proteins independent of their molecular weight, importantly, WGA-induced inhibition of nucleocytoplasmic transport has been shown to be specific for facilitated transport processes (Dabauvalle *et al.,* 1988). WGA does not simply block the NPC, preventing all transport. Passive diffusion of small fluorescently labelled dextran molecules has been shown to proceed at the same rate through the NPC in the presence and absence of WGA (Yoneda *et al.,* 1987; Dabauvalle *et al.,* 1988; Wolff *et al.,* 1988). Transport of a range of molecules in *Xenopus* oocytes has proved sensitive to WGA inhibition. These include import of the U1-U6 snRNAs (Fischer *et al.,* 1991; Michaud and Goldfarb, 1992), 5S RNA and ribosomal protein L5 (Allison *et al.,* 1993; Murdoch and Allison, 1996), calmodulin (Pruschy *et al.,* 1994), and histone H1 (Kurz *et al.,* 1997), and the export of ribosomal subunits (Bataillé *et al.,* 1990) and signal recognition particle RNA (He *et al.,* 1994).

3.1.8 Summary of results

This chapter addresses the nucleocytoplasmic transport of thyroid hormone receptor isoforms, α and β, as well as the viral oncogenic variant, v-ErbA, and the *in vitro*-generated DNA-binding mutant, C122A, in *Xenopus* oocytes. Import and export were assessed in

oocytes by microinjection of [^{35}S]-methionine-labelled protein. The results presented in this chapter demonstrate unexpected and novel findings.

Import of TR and variants was assessed by chilling, competition, WGA, and apyrase (ATP depletion) assays in oocytes, whilst chilling assays alone were performed for export. Import of both TR isoforms and mutant C122A was shown to occur by a passive diffusion. However, import of ribosomal protein L5, a protein known to be facilitatively transported, was inhibited by chilling, energy depletion, lectin binding, and competition assays. In contrast to import, export of TRα, TRβ, and C122A was temperature dependent, indicating that efflux of these receptors was not by diffusion. In addition, import and export of v-ErbA (which is larger in size due to its fusion to viral *gag* protein), was inhibited by chilling, competition, WGA, and ATP depletion.

Transport of EGFP-tagged receptors in transfected mammalian cells, was also tested. These receptors, whilst being larger than the unlabelled receptor molecules due to the fusion with GFP protein, were expressed in the mammalian cell system (as presented in Section 2.3.3). TRα::EGFP remained nuclear during chilling and energy depletion assays, suggesting that import of receptor synthesised before commencement of these assays was not inhibited (or was not at a detectable level) and that export of receptor by passive diffusion was not occurring. C122A remains cytoplasmic, even under conditions of chilling and ATP depletion. Potential reasons for the difference in observations between these two systems is discussed, as well as the implications of the passive transport mechanism observed for TR in *Xenopus* oocytes.

3.2 Materials and Methods

3.2.1 Plasmids

The plasmids used in the determination of receptor distribution in *Xenopus* oocytes were also utilised for *Xenopus* nucleocytoplasmic transport studies. A description of these plasmids, pBS-rTRα1, pGEM-TRβ1, pGEM-C122A, pGEM-v-*erbA*, and pSP6-L5, is contained in Section 2.2.1.

The EGFP fusion plasmids for TRα and C122A, as well as wild-type EGFP, described in Section 2.2.1 for the receptor distribution studies in transfected mammalian cells, were also used in these nucleocytoplasmic transport studies. EGFP fusion plasmids for TRβ and v-ErbA were not used in these transport studies, as they could not be generated, as discussed in Section 2.2.1.

3.2.2 Characterisation of receptor transport in Xenopus oocytes

In vitro synthesised proteins were prepared, by coupled transcription/translation in rabbit reticulocyte lysate (Promega) supplemented with [^{35}S]-methionine (Life Technologies), for transport studies in *Xenopus* oocytes, as described in Section 2.2.2. Oocytes were surgically removed from female adult *Xenopus laevis* and prepared for experimentation as described in Section 2.2.3. Microinjections were performed as described in Section 2.2.3 and oocytes treated as described in the assays below. Treatments for experiments performed in *Xenopus* oocytes are summarised in Table 10.

3.2.3 Chilling assays in Xenopus oocytes

To assess temperature dependence, oocytes pre-chilled on ice, at 0-4°C for 30 minutes, were microinjected with *in vitro* synthesised protein. Oocytes were not maintained on ice during the injection process, and therefore the number of oocytes injected at one time was reduced to give a maximum time of three minutes away from chilled conditions. Post-injection, oocytes were incubated on ice at 0-4°C, for six hours. As controls to ensure that each batch of oocytes was metabolically active, injected oocytes were also incubated at 18-20°C for the same period, or for six hours on ice followed by six hours at 18-20°C, in order to determine whether physiological temperatures could restore transport competency. After incubation, nuclei were dissected in ice-cold NIM and extracted protein was assayed for nuclear import, as described in Section 2.2.4.

3.2.4 ATP depletion and WGA assays in Xenopus oocytes

ATP depletion, WGA, and competition assays were only performed for import in *Xenopus* oocytes, due to deterioration in the supply of healthy *Xenopus* frogs and subsequently their oocytes. Relocation of the frogs to a vivarium containing mice and rats, as well as a change in the frequency and type of diet, was followed by susceptibility of the *Xenopus* to several fungal and nematode infections. Experimentation was not undertaken during treatment of infections, and limited replicate experiments were performed after the relocation. ATP depletion assays were performed according to existing laboratory protocols with some modification (Allison *et al.,* 1993; Murdoch and Allison, 1996). Oocytes were preinjected with apyrase (Grade VIII, Sigma Chemical Co.) 30 minutes prior to microinjection of *in vitro* synthesised proteins. Fifty nanolitres of 1 U/μl apyrase, dissolved in PBS, was delivered to give an intracellular concentration of 100 U/ml, which has been shown to deplete ATP in a previous study undertaken in this laboratory (Allison *et al.,* 1993). After six hours incubation at 18-20°C, oocytes were manually dissected and assayed as described in Section 2.2.4.

Wheat germ agglutinin assays were also carried out according to established laboratory protocols, with the modification of buffers as described above (Allison *et al.,* 1993;

Murdoch and Allison, 1996). Fifty nanolitres of WGA (lectin, from *Triticum vulgaris*; Sigma) dissolved in PBS, or PBS as a control, were injected to give a final intracellular concentration of 500 μg/ml, three hours prior to microinjection of protein. After introduction of protein and incubation for six hours at 18-20°C, oocytes were assayed for nuclear import.

3.2.5 Histone H1 and ribosomal protein L5 competition assays in Xenopus oocytes

Two concentrations of histone H1 (Boehringer Mannheim) were initially trialled for inhibition of protein L5 facilitated import, based upon the concentration of 810 μM used by Breeuwer and Goldfarb (1990). Histone H1 was microinjected into oocytes to give final intracellular concentrations of 750 μM and 900 μM (designated low histone H1, LH1 or [low], and high histone H1, HH1 or [high], respectively). The effect of the introduced histone H1 was assessed to determine whether the concentrations fell within an inhibitory range, by dissection of oocytes after six hours incubation at 18-20°C and analysis as detailed in Section 2.2.4. Subsequently, these concentrations were used in the studies of TRα, TRβ, C122A, and v-ErbA.

Ribosomal L5 was also used as a competitor in experiments similar to those performed with histone H1. Equal quantities of [^{35}S]-methionine-labelled L5 and unlabelled L5 (1 ng of each per 50 nl delivery) were introduced into each *Xenopus* oocyte. The concentration of unlabelled L5 in the translation mix was determined by comparison with labelled L5. The two forms of L5 were separated by PAGE and visualised by Coomassie Blue staining, as unlabelled L5 could not be detected by autoradiography. The gels were incubated in five volumes of stain (0.25% Coomassie brilliant blue R-250; 50% methanol; 10% acetic acid) for four hours with shaking, at room temperature. After the removal of the stain, the gel was destained by successive incubations in destain solution (5% methanol; 7.5% acetic acid), prior to photography with a Nikon 35 mm camera. The subcellular distribution of microinjected, [^{35}S]-labelled L5, with and without coinjection of unlabelled L5, was determined by analysis and autoradiography as described in Section 2.2.4. Subsequent coinjections of labelled L5 were made with TRα, TRβ, C122A, and v-ErbA, prior to analysis as described (Section 2.2.4).

Table 10 - Transport inhibition treatments in *Xenopus* oocytes.

Treatment	*Effect*	*Incubation*
Wheat germ agglutinin	Inhibits facilitated transport, binds GlcNAc-NPC proteins	Three hours preinjection, six hours at 18-20°C
Chilling	Inhibits facilitated transport	Six hours at 0-4°C Six hours at 0-4°C/18-20°C
Apyrase	Inhibits facilitated transport, ATP depletion	Thirty minutes preinjection, six hours at 18-20°C
Histone H1 (H1)	Competitor for general facilitated protein import pathway	Coinjection, six hours at 18-20°C

3.2.6 Chilling and ATP depletion assays in cultured mammalian cells

NIH 3T3 cell cultures were maintained and prepared for experimentation as described in Section 2.2.7. Transfections were performed with the EGFP fusion vectors, EGFP::TRα and EGFP::C122A as well as wild type EGFP, as described in Section 2.2.8. The incubation medium was removed 24-48 hours post-transfection and cells were washed in D-PBS. The medium was replaced with medium containing 50 μg/ml cycloheximide for 30 minutes to prevent protein synthesis. This was necessary to ensure that any cytoplasmic protein observed was not freshly synthesised, and had therefore had insufficient time to be localised. A further replacement was made with cycloheximide-containing medium, as detailed in Table 11, and cells were incubated at 37°C/5% CO_2:95% air, or 0°C as shown, for the time indicated prior to visualisation. Glucose minus DMEM, with a non-hydrolysable analogue of glucose, 2-deoxyglucose (Sigma), and a metabolic inhibitor, oligomycin (types A, B, and C; Sigma), were used in energy depletion experiments. Cells were observed under bright field, fluorescence, and a combination of both conditions with a Leitz Orthoplan fluorescence microscope (as described in Section 2.2.9).

Table 11 - Transport assays in cultured NIH/3T3 cells.

Assay	*Culture medium*	*Treatment*	*Incubation*
ATP depletion	DMEM (pyruvate/glucose minus)	6 mM 2-deoxyglucose 50 μM oligomycin	Four hours
ATP control	complete DMEM	37°C	Four hours
Chilling	complete DMEM	0-4°C	Three hours
Chilling control	complete DMEM	37°C	Three hours

3.3 Results

The following results are presented separately according to the cell type in which the experiments were performed. Sections 3.3.1 to 3.3.5 describe the results of transport studies carried out in microinjected *Xenopus* oocytes. The results of low temperature incubations on import and export of TRα (46.8 kDa), TRβ (52 kDa), C122A (52 kDa), and v-ErbA (75 kDa) are presented in Sections 3.3.1 and 3.3.2 (and jointly displayed in Figures 3.1 to 3.5). Results for the competition assays with ribosomal protein L5 and histone H1 are given in Sections 3.3.4 and 3.3.3 (and jointly displayed in Figures 3.6 to 3.10), whilst ATP depletion and WGA inhibition assays are presented in Section 3.3.5. Data obtained from the transfection of NIH 3T3 cells with EGFP-tagged TRα and C122A are displayed in Sections 3.3.6 and 3.3.7. The former describes the results for low temperature incubations and the latter contains the results for ATP depletion studies in NIH 3T3 cells. Results for nucleocytoplasmic transport studies for *Xenopus* oocytes and cultured NIH 3T3 cells are summarised in Table 13 and Table 14, respectively. These tables also contain the probability values for t-tests carried out between data sets.

3.3.1 Import of TRα, TRβ, and C122A in Xenopus oocytes is temperature independent

Facilitated transport is inhibited in chilled cells whilst passive diffusion is not (described in Section 3.1.5). Therefore, to determine through which mechanisms nuclear import of TR and its variants occur, pre-chilled *Xenopus* oocytes were cytoplasmically injected with *in vitro* synthesised [^{35}S]-methionine-labelled receptor molecules. A further incubation at 0-4°C was carried out for five to six hours post-injection to determine whether receptor transport was inhibited by low temperature incubations. As a control to assess the continuing viability of the cell environment, the chilled oocytes were returned to physiological temperatures after low temperature incubations to determine whether nuclear import was restored for the proteins affected by chilling.

Surprisingly, nuclear import of TRα did not appear to be inhibited by incubation of oocytes on ice at 0-4°C (Figure 3.1). Analysis by student's t-tests confirmed this finding ($p>0.1$). Import of TRβ and C122A was also not significantly inhibited ($p>0.1$) by chilling (Figures 3.2 and Figure 3.3). Although no significant inhibition of v-ErbA import by chilling was observed, great variability was observed between batches of oocytes (Figure 3.4). It was observed that the nuclear integrity of chilled oocytes microinjected with v-ErbA was not maintained to the extent of other oocytes. In addition, as such a low level of import was observed, it was difficult to assess significant differences between treatments. Ribosomal protein L5 was used as a control in these studies, as L5 has been shown to be imported to *Xenopus* nuclei by a facilitated process (Murdoch and Allison, 1996). Inhibition of ribosomal protein L5 nuclear import by chilling was observed as expected (Figure 3.5). Analysis by Student's t-tests supported this inhibition effect ($p<0.001$). Competence of L5 import was restored when oocytes were further incubated for six hours at 18-20°C (Figure 3.5 and Table 13).

3.3.2 Chilling inhibits TRα export in Xenopus oocytes

Due to the size of *Xenopus* oocytes it is possible to microinject *in vitro* synthesised proteins directly into the nuclear compartment, in order to investigate their nuclear export characteristics. As nuclear export of TR has not been demonstrated prior to the investigations contained in this thesis (Section 2.3.2), TRα and TRβ, C122A, v-ErbA, and L5 were introduced into *Xenopus* oocytes and export was assessed.

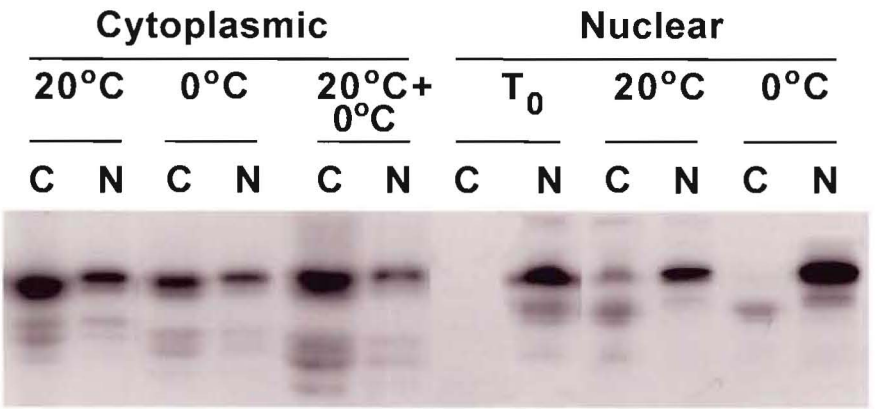
Approximately 62% of TRα was retained in the nuclear compartment of *Xenopus* oocytes (Figure 3.1), indicating that export of a subpopulation of TR (38%) can occur. A proportion of TRβ and C122A was also exported, showing cytoplasmic distributions of 26% and 28% respectively, as presented in Figures 3.2 and 3.3. However, v-ErbA was retained in the nucleus to a greater extent as shown in Figure 3.4. Approximately 23% of v-ErbA was localised to the cytoplasmic fractions of *Xenopus* oocytes.

Figure 3.1. ***The effect of chilling on the nuclear import of TRα in Xenopus oocytes.***

A. Approximately 100 picograms of *in vitro* synthesised [^{35}S]-methionine labelled TRα were introduced into each oocyte by microinjection. Oocytes were incubated at 18-20°C for five to six hours prior to manual dissection of the oocytes into cytoplasmic (**C**) and nuclear (**N**) fractions. As a control, oocytes were returned to physiological temperatures after low temperature incubations to determine whether nuclear import was restored for the proteins affected by chilling (**0°C+20°C**). Protein was isolated from six pooled nuclear or cytoplasmic fractions and separated from oocyte proteins by electrophoresis on 12% discontinuous polyacrylamide gels containing SDS, followed by autoradiography. (**T_0**: initial time point performed after microinjection). A representative autoradiogram is shown.

B. The percentages of nuclear accumulation from the experiments described above were determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of replicate batches (**n**) of six pooled oocytes. The bars indicate standard error of the means. (TRα[20°C]: n=8; TRα[0°C]: n=4; TRα[0°C+20°C]: n=3; TRα[20°C/nu.]: n=8; TRα[0°C/nu.]: n=4). (**nu.**: nuclear injections)

A


Cytoplasmic
Nuclear
20°C
0°C
20°C+ 0°C
T0
20°C
0°C
C N C N C N C N C N C N

B

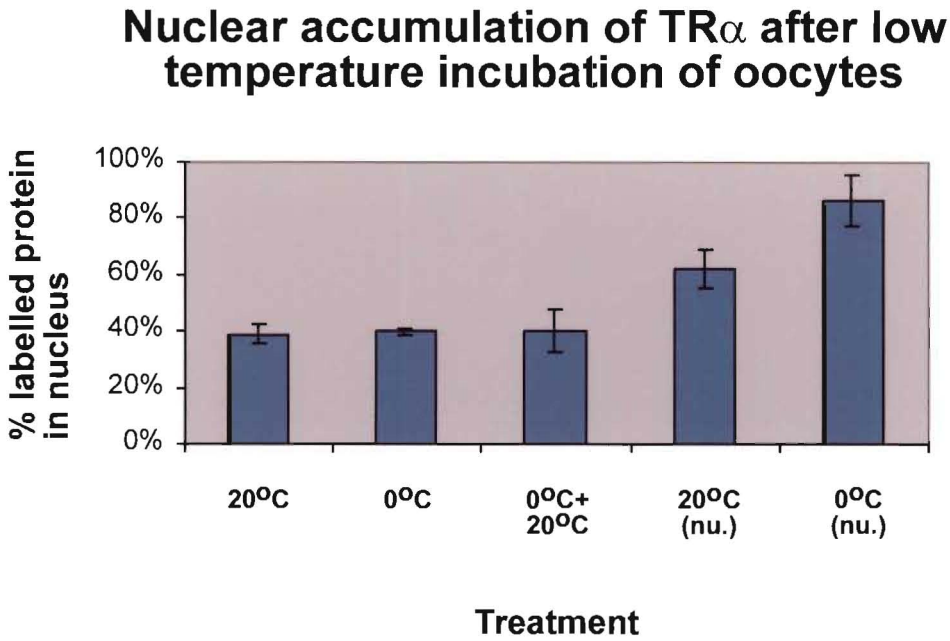
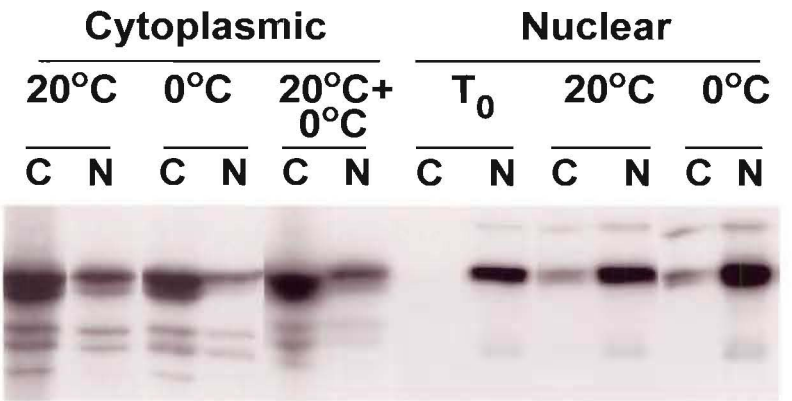

Nuclear accumulation of TRα after low temperature incubation of oocytes
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
20°C
0°C
0°C+ 20°C
20°C (nu.)
0°C (nu.)
Treatment

Figure 3.2. ***The effect of chilling on the nuclear import of TRβ in Xenopus oocytes.***

A. Oocytes were microinjected with [^{35}S]-methionine labelled TRβ. Five to six hours after incubation at 18-20°C, protein was isolated from oocytes cytoplasmic (**C**) and nuclear (**N**) fractions, and analysed as described in Figure 3.1. As a control, oocytes were returned to physiological temperatures after low temperature incubations (**0°C+20°C**) to determine whether nuclear import was restored for the proteins affected by chilling, prior to dissection. ($\mathbf{T_0}$: initial time point performed after microinjection). A representative autoradiogram is shown.

B. The percentages of nuclear accumulation from replicate experiments were determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of replicate batches (**n**) of pooled oocytes. The bars indicate standard error of the means. (TRβ[20°C]: n=4; TRβ[0°C]: n=4; TRβ[0°C+20°C]: n=3; TRβ[20°C/nu.]: n=3; TRβ[0°C/nu.]: n=3) (**nu.**: nuclear injections)

A


Cytoplasmic
Nuclear
20°C
0°C
20°C+ 0°C
T0
20°C
0°C
C N C N C N C N C N C N

B

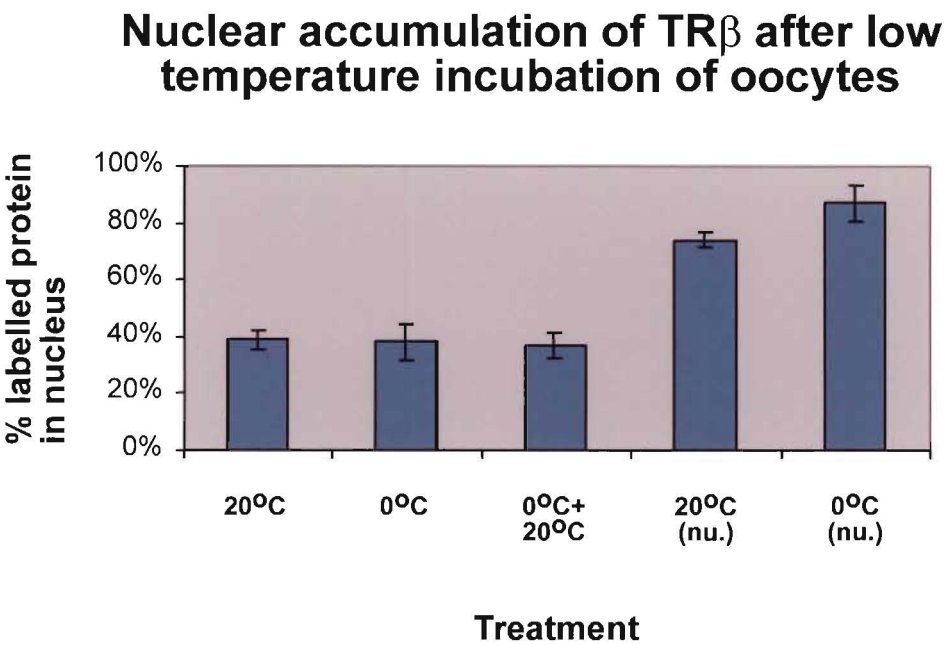
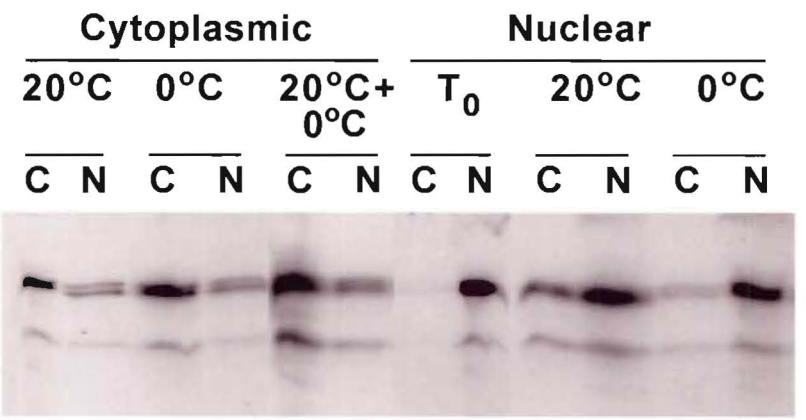

Nuclear accumulation of TRβ after low temperature incubation of oocytes
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
20°C
0°C
0°C+ 20°C
20°C (nu.)
0°C (nu.)
Treatment

Figure 3.3. ***The effect of chilling on the nuclear import of C122A in Xenopus oocytes.***

A. Oocytes were microinjected with [^{35}S]-methionine labelled C122A. Five to six hours after incubation at 18-20°C, protein was isolated from oocytes cytoplasmic (**C**) and nuclear (**N**) fractions, and analysed as described in Figure 3.1. As a control, oocytes were returned to physiological temperatures after low temperature incubations (**0°C+20°C**) to determine whether nuclear import was restored for the proteins affected by chilling, prior to dissection. ($\mathbf{T_0}$: initial time point performed after microinjection). A representative autoradiogram is shown.

B. The percentages of nuclear accumulation from replicate experiments were determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of replicate batches (**n**) of pooled oocytes. The bars indicate standard error of the means. (C122A[20°C]: n=4; C122A[0°C]: n=4; C122A[0°C+20°C]: n=3; C122A[20°C/nu.]: n=3; C122A[0°C/nu.]: n=2) (**nu.**: nuclear injections)

A


Cytoplasmic
Nuclear
20°C
0°C
20°C+ 0°C
T0
20°C
0°C
C N C N C N C N C N C N

B

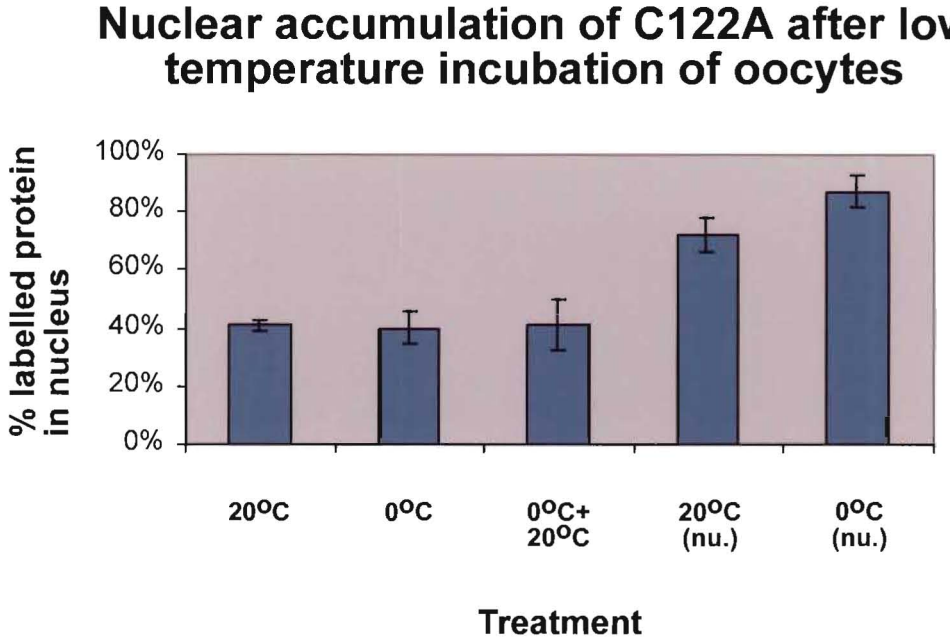
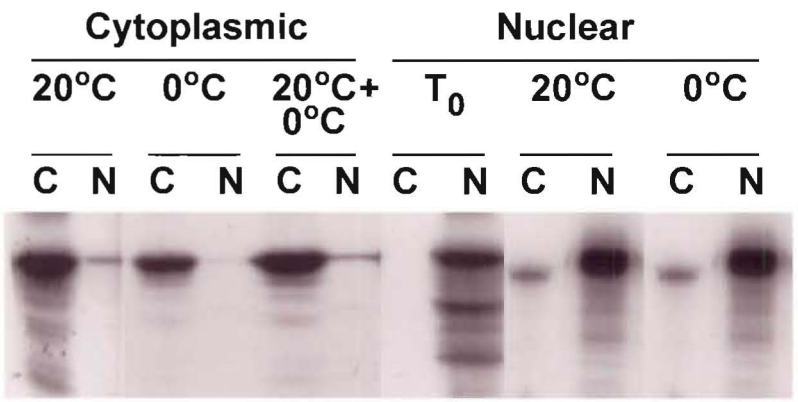

Nuclear accumulation of C122A after low temperature incubation of oocytes
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
20°C
0°C
0°C+ 20°C
20°C (nu.)
0°C (nu.)
Treatment

Figure 3.4. ***The effect of chilling on the nuclear import of v-ErbA in Xenopus oocytes.***

A. Oocytes were microinjected with [^{35}S]-methionine labelled v-ErbA. Five to six hours after incubation at 18-20°C, protein was isolated from oocytes cytoplasmic (**C**) and nuclear (**N**) fractions, and analysed as described in Figure 3.1. As a control, oocytes were returned to physiological temperatures after low temperature incubations (**0°C+20°C**) to determine whether nuclear import was restored for the proteins affected by chilling, prior to dissection. ($\mathbf{T_0}$: initial time point performed after microinjection). A representative autoradiogram is shown.

B. The percentage of labelled protein in the oocyte nuclei from replicate experiments was quantified by densitometry of the autoradiograms produced in the experiments described in A. Each graphed data point reflects the mean of replicate batches (**n**) of pooled oocytes. The bars indicate standard error of the means. (v-ErbA[20°C]: n=9; v-ErbA[0°C]: n=4; v-ErbA[20°C+0°C]: n=3; v-ErbA[20°C/nu.]: n=5; v-ErbA[0°C/nu.]: n=4) (**nu.**: nuclear injections)

A


Cytoplasmic
Nuclear
20°C
0°C
20°C+ 0°C
T0
20°C
0°C
C N C N C N C N C N C N

B

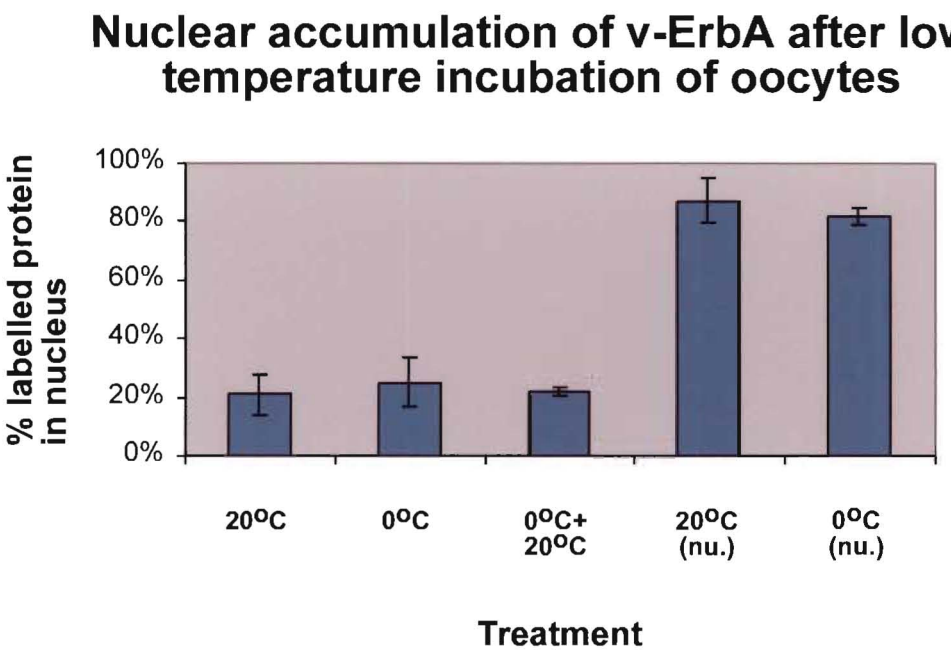
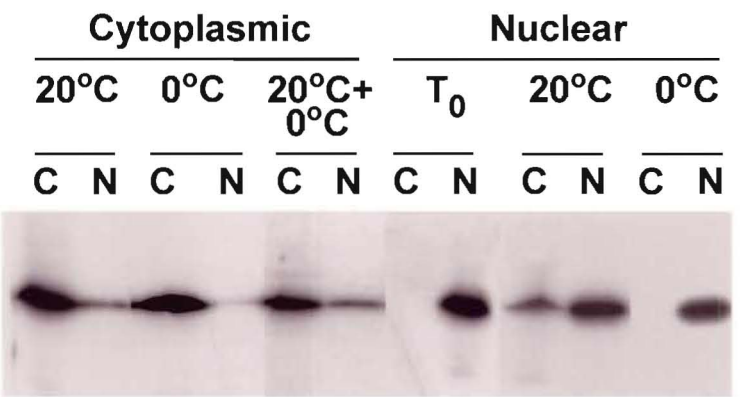

Nuclear accumulation of v-ErbA after low temperature incubation of oocytes
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
20°C
0°C
0°C+ 20°C
20°C (nu.)
0°C (nu.)
Treatment

Figure 3.5. ***The effect of chilling on the nuclear import of L5 in Xenopus oocytes.***

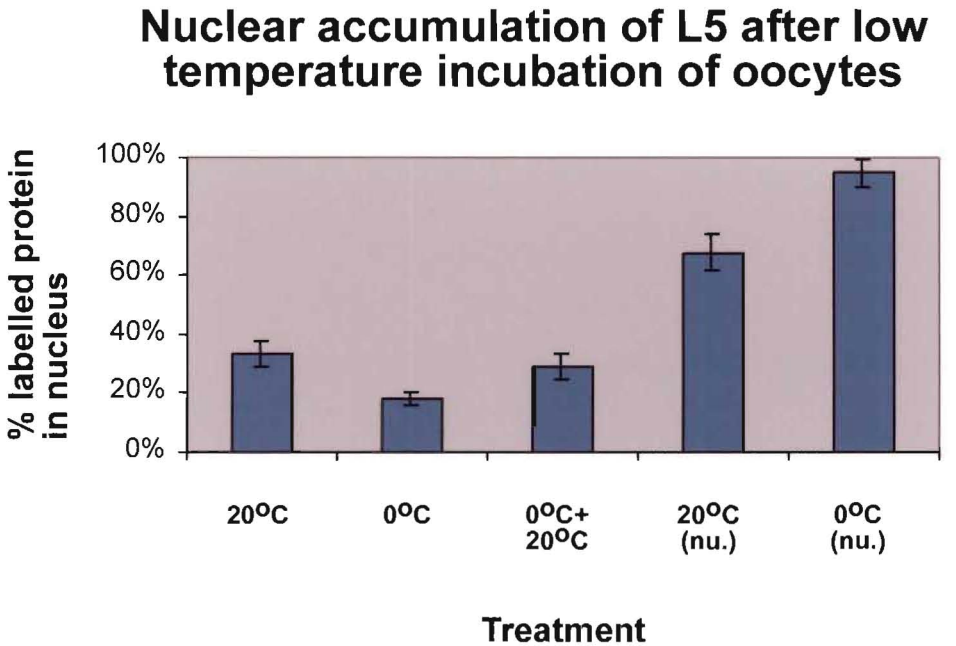
A. Oocytes were microinjected with [^{35}S]-methionine labelled L5. Five to six hours after incubation at 18-20°C, protein was isolated from oocytes cytoplasmic (**C**) and nuclear (**N**) fractions, and analysed as described in Figure 3.1. As a control, oocytes were returned to physiological temperatures after low temperature incubations (**0°C+20°C**) to determine whether nuclear import was restored for the proteins affected by chilling, prior to dissection. ($\mathbf{T_0}$: initial time point performed after microinjection). A representative autoradiogram is shown.

B. The percentage of labelled protein in the oocyte nuclei from replicate experiments was quantified by densitometry of the autoradiograms produced in the experiments described in A. Each graphed data point reflects the mean of replicate batches (**n**) of pooled oocytes. The bars indicate standard error of the means. (L5[20°C]: n=4; L5[0°C]: n=5; L5[0°C+20°C]: n=3; L5[20°C/nu.]: n=4; L5[0°C/nu.]: n=3) (**nu.**: nuclear injections)

A


Cytoplasmic
Nuclear
20°C
0°C
20°C+ 0°C
T0
20°C
0°C
C N C N C N C N C N C N

B


Nuclear accumulation of L5 after low temperature incubation of oocytes
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
20°C
0°C
0°C+ 20°C
20°C (nu.)
0°C (nu.)
Treatment

A subpopulation of ribosomal protein L5 (32%) was also exported from oocyte nuclei (Figure 3.5). It would be expected that a population of L5 would remain nuclear as L5 has been implicated in the nuclear import of 5S RNA (in a complex termed 5S RNP) and in nuclear retention of 5S RNA in the oocyte nucleoli (Allison *et al.,* 1993; Murdoch and Allison, 1996; North and Allison, 1997).

The mechanism of nuclear export of the receptors was assessed by low temperature incubations. Replicate results and statistical analysis are presented in Table 13. TRα, TRβ, and C122A display similar export characteristics (Figures 3.1, 3.2, and 3.3). 86% of TRα is retained in the nucleus and 87% of both TRβ and C122A. Although export continues at low temperatures, the process is significantly inhibited in all cases ($p<0.001$ $p=0.03$, and $p=0.06$, respectively). Therefore, nuclear export of TRα, TRβ, and C122A is temperature dependent. Inhibition of v-ErbA export at low temperatures was not observed for all batches of oocytes. In fact an increased amount of receptor (82%) is exported. As reported in Section 3.3.1, integrity of the nuclear membrane appeared somewhat compromised in chilled oocytes injected with v-ErbA. This is not unexpected as v-ErbA has been observed to induce nuclear breakdown in *Xenopus* oocytes at physiological temperatures, although this took at least 24 hours to be initiated (Nagl *et al.,* 1997). Lastly, L5 export is temperature dependent (Figure 3.5) and is therefore inhibited by low temperature incubations ($p<0.001$).

3.3.3 Histone H1 does not inhibit TRα, TRβ, or C122A import in Xenopus oocytes

As an additional criterion for characterising import of TRα, TRβ, C122A, and v-ErbA, competition assays with histone H1 and ribosomal protein L5 were performed. Combined results for these competition assays are presented in Figures 3.6 to 3.10. The rationale for these competition experiments is that import kinetics of the introduced TR variants would be at a reduced level if histone H1 or L5, acting as direct competitors of facilitated processes, were imported by the same mechanisms as TRα, TRβ, C122A, or v-ErbA. This reduction is due to competition for shared transport factors; that is, facilitated transport is saturable.

An excess of histone H1 has been used previously to investigate import of proteins (Breeuwer and Goldfarb, 1990). If TRα, TRβ, C122A, or v-ErbA were imported by a facilitated or active mechanism, a decrease in observed nuclear localisation would be expected after incubation for six hours.

In order to ascertain histone H1 concentrations at which inhibition of facilitated transport occurred, excess histone H1 was competed against ribosomal L5 (0.1 ng) at two intracellular concentrations of 750 μM and 900 μM. Both concentrations were shown to inhibit the nuclear import of L5 ($p<0.001$), as demonstrated in Figure 3.6 (A and D) and Table 13.

TRα import was not inhibited ($p>0.1$) by coinjection with histone H1 (Figure 3.7A), at both low and high concentrations of histone H1. The excess of histone H1 did not influence TRβ import (Figure 3.8, A and C), nor did it affect import of C122A (Figure 3.9, A and C), at low ($p>0.1$) or high histone H1 levels ($p>0.1$). Coinjection with the higher concentration of histone H1 resulted in inhibition of v-ErbA import ($p=0.05$), supporting a facilitated or active mechanism of nuclear transport (Figure 3.10, A and C).

3.3.4 Competition with ribosomal protein L5 does not affect TRα, TRβ, or C122A import in Xenopus oocytes

Import of L5 is via a facilitated process and would therefore be expected to have similar competition effects to histone H1. As is the case with the histone H1 assays (Section 3.3.3), coinjection of a protein with the same transport pathway requirements would lead to a reduction in the percentage of the respective proteins being localised to the *Xenopus* oocyte nucleus.

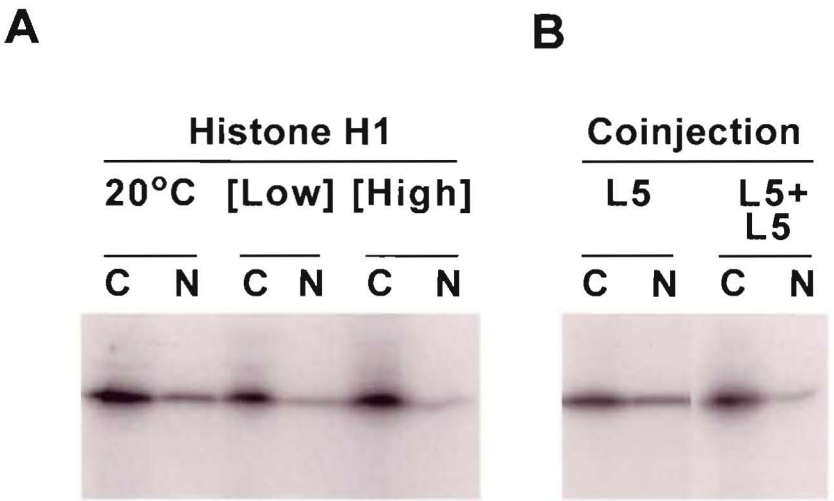
Figure 3.6. ***The effect of competing proteins on the nuclear import of L5 in <u>Xenopus</u> oocytes.***

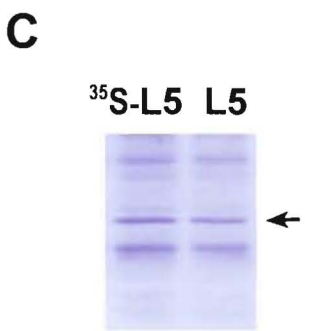
A. Oocytes were microinjected with 100 picograms of [^{35}S]-methionine labelled ribosomal protein L5 and histone H1 (to an intracellular concentration of 750 μM or 900 μM). Five to six hours after incubation at 18-20°C, protein was isolated from oocytes cytoplasmic (**C**) and nuclear (**N**) fractions, and analysed as described in Figure 3.1. (**LH1** or **[low]**: low histone H1 concentration of 750 μM; **HH1** or **[high]**: high histone H1 concentration of 900 μM. A representative autoradiogram is shown.

B. Oocytes were microinjected with 100 picograms of [^{35}S]-methionine labelled ribosomal protein L5 and 100 picograms of unlabelled protein L5 and analysed as described in **A.**

C. Coomassie blue stained gel of translation products produced *in vitro*. The staining indicates that equimolar amounts of [^{35}S]-methionine-labelled and unlabelled ribosomal protein L5 were obtained. Equal volumes of protein were used from each sample to compete each other in the experiments described in **B**.

D. The percentages of nuclear accumulation from replicate experiments were determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of replicate batches (**n**) of pooled oocytes, as described in Figure 3.1. Standard error of the means is indicated by the bars. (L5[20°C]: n=4; L5[LH1]: n=3; L5[HH1]: n=3; L5+L5: n=2)


A
B
Histone H1
20°C [Low] [High]
C N C N C N
Coinjection
L5
L5+ L5
C N C N


C
35S-L5 L5

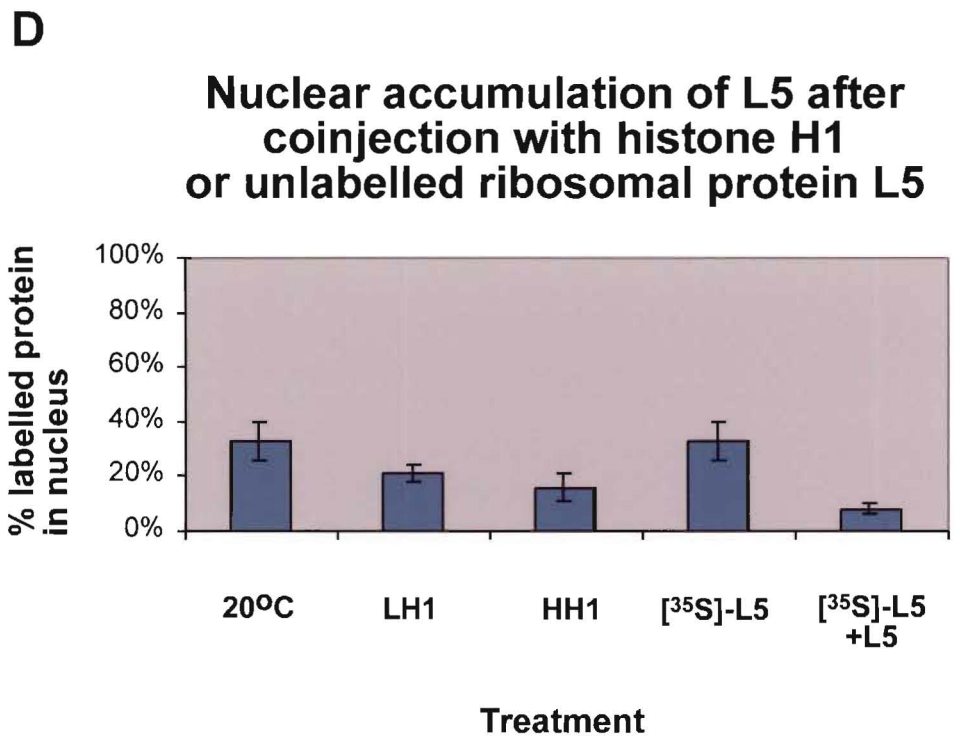

D
Nuclear accumulation of L5 after coinjection with histone H1 or unlabelled ribosomal protein L5
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
20°C
LH1
HH1
[35S]-L5
[35S]-L5 +L5
Treatment

Figure 3.7. ***The effect of competing proteins on the nuclear import of TRα in Xenopus oocytes.***

A. Oocytes were microinjected with 100 picograms of [^{35}S]-methionine labelled TRα and histone H1 (to an intracellular concentration of 750 μM or 900 μM). Five to six hours after incubation at 18-20°C, protein was isolated from oocytes cytoplasmic (**C**) and nuclear (**N**) fractions, and analysed as described in Figure 3.1. (**LH1** or **[low]**: low histone H1 concentration of 750 μM; **HH1** or **[high]**: high histone H1 concentration of 900 μM). A representative autoradiogram is shown.

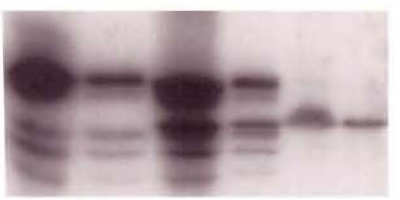
B. Oocytes were microinjected with 100 picograms of [^{35}S]-methionine labelled TRα and 100 picograms of [^{35}S]-methionine labelled ribosomal protein L5 and analysed as described in **A.**

C. The percentages of nuclear accumulation from replicate experiments were determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of replicate batches (**n**) of pooled oocytes, as described in Figure 3.1. Standard error of the means is indicated by the bars. (TRα[20°C]: n=8; TRα[LH1]: n=4; TRα[HH1]: n=6; TRα+L5: n=3; L5: n=4)

A


Histone H1
20°C [Low] [High]
C N C N C N

B


Coinjection
TRα
L5+ TRα
L5
C N C N C N

C

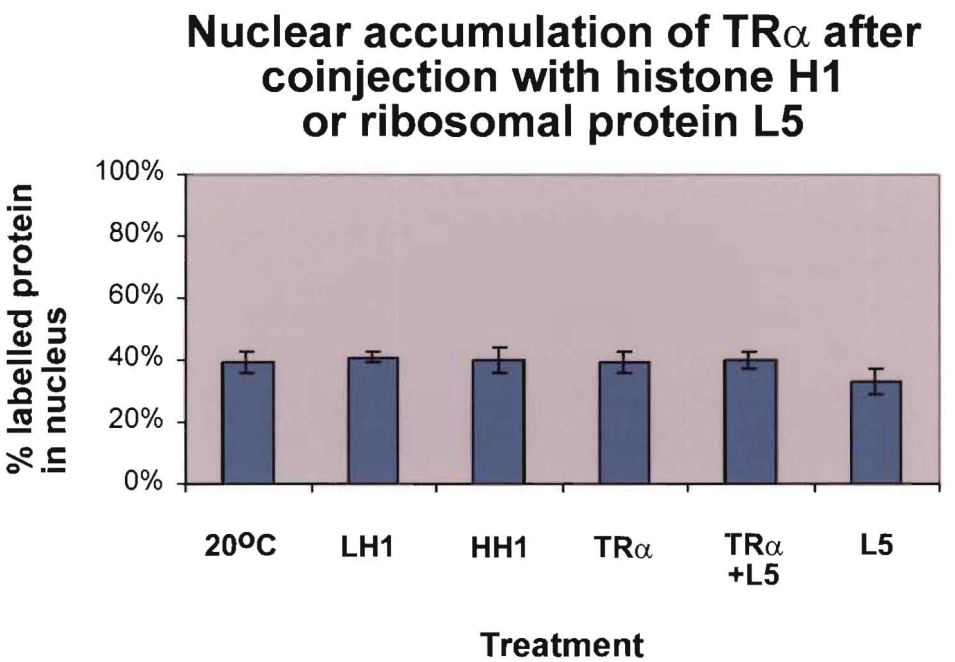

Nuclear accumulation of TRα after coinjection with histone H1 or ribosomal protein L5
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
20°C
LH1
HH1
TRα
TRα +L5
L5
Treatment

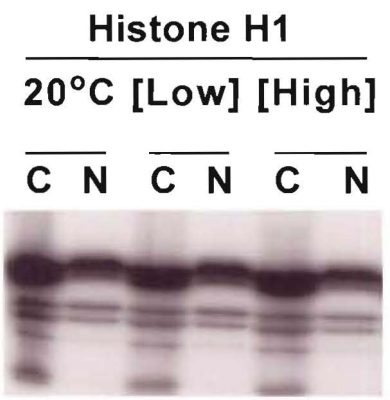
Figure 3.8. ***The effect of competing proteins on the nuclear import of TRβ in Xenopus oocytes.***

A. Oocytes were microinjected with 100 picograms of [^{35}S]-methionine labelled TRβ and histone H1 (to an intracellular concentration of 750 μM or 900 μM). Five to six hours after incubation at 18-20°C, protein was isolated from oocytes cytoplasmic (**C**) and nuclear (**N**) fractions, and analysed as described in Figure 3.1. (**LH1** or **[low]**: low histone H1 concentration of 750 μM; **HH1** or **[high]**: high histone H1 concentration of 900 μM)

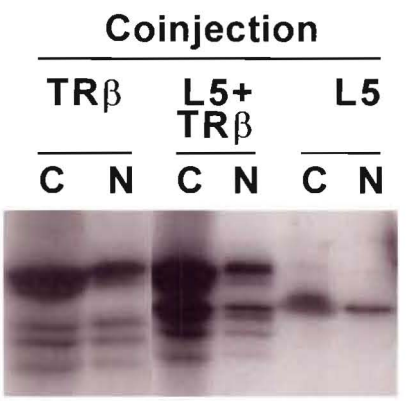
B. Oocytes were microinjected with 100 picograms of [^{35}S]-methionine labelled TRβ and 100 picograms of [^{35}S]-methionine labelled ribosomal protein L5 and analysed as described in **A.**

C. The percentages of nuclear accumulation from replicate experiments were determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of replicate batches (**n**) of pooled oocytes, as described in Figure 3.1. Standard error of the means is indicated by the bars. (TRβ[20°C]: n=4; TRβ[LH1]: n=2; TRβ[HH1]: n=2; TRβ+L5: n=3; L5: n=4)

A


Histone H1
20°C [Low] [High]
C N C N C N

B


Coinjection
TRβ
L5+ TRβ
L5
C N C N C N

C

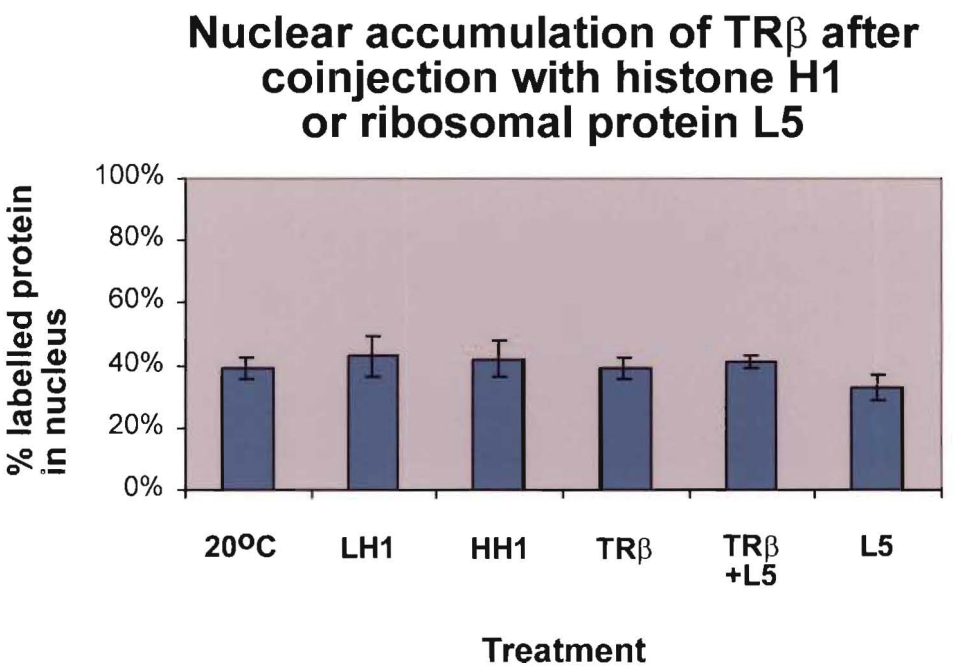

Nuclear accumulation of TRβ after coinjection with histone H1 or ribosomal protein L5
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
20°C
LH1
HH1
TRβ
TRβ +L5
L5
Treatment

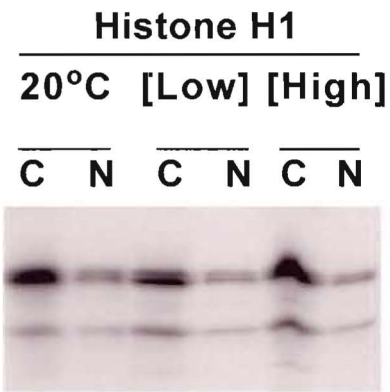
Figure 3.9. ***The effect of competing proteins on the nuclear import of C122A in Xenopus oocytes.***

A. Oocytes were microinjected with 100 picograms of [^{35}S]-methionine labelled C122A and histone H1 (to an intracellular concentration of 750 μM or 900 μM). Five to six hours after incubation at 18-20°C, protein was isolated from oocytes cytoplasmic (**C**) and nuclear (**N**) fractions, and analysed as described in Figure 3.1. (**LH1** or **[low]**: low histone H1 concentration of 750 μM; **HH1** or **[high]**: high histone H1 concentration of 900 μM)

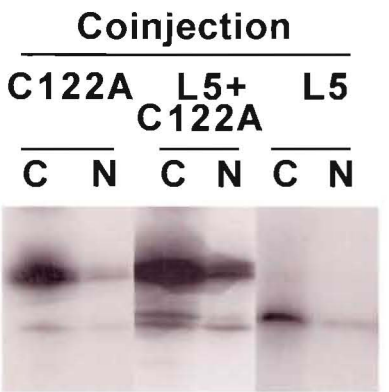
B. Oocytes were microinjected with 100 picograms of [^{35}S]-methionine labelled C122A and 100 picograms of [^{35}S]-methionine labelled ribosomal protein L5 and analysed as described in **A.**

C. The percentages of nuclear accumulation from replicate experiments were determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of replicate batches (**n**) of pooled oocytes, as described in Figure 3.1. Standard error of the means is indicated by the bars. (C122A[20°C]: n=4; C122A[LH1]: n=3; C122A[HH1]: n=3; C122A+L5: n=3; L5: n=4)

A


Histone H1
20°C [Low] [High]
C N C N C N

B


Coinjection
C122A L5+ C122A L5
C N C N C N

C

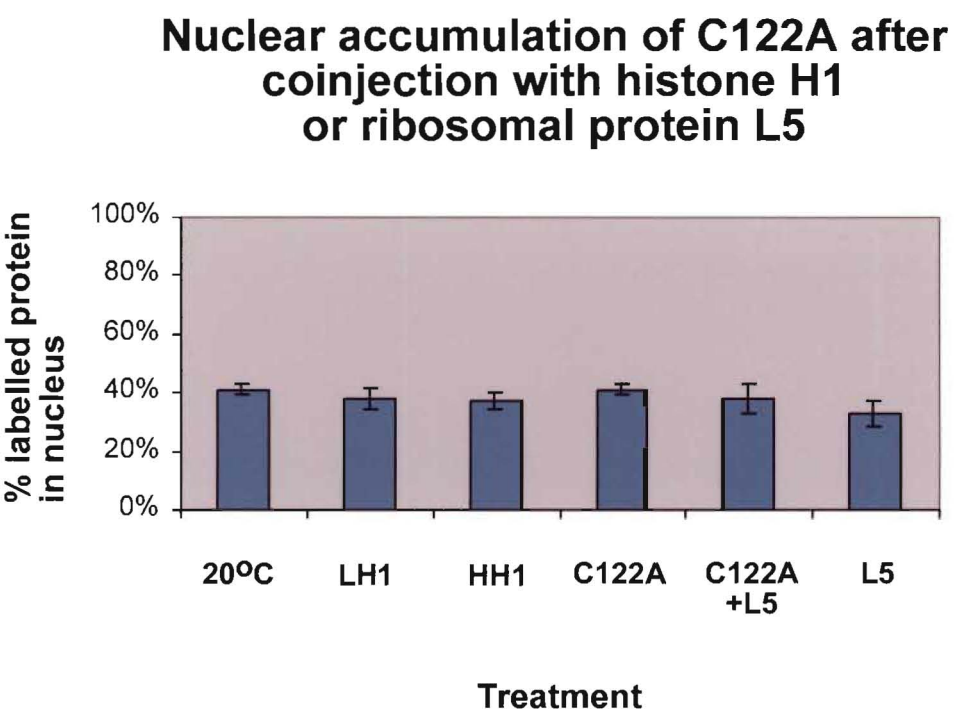

Nuclear accumulation of C122A after coinjection with histone H1 or ribosomal protein L5
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
20°C
LH1
HH1
C122A
C122A +L5
L5
Treatment

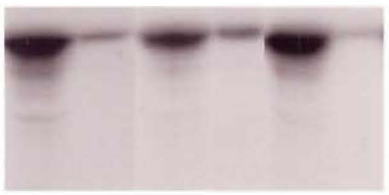
Figure 3.10. ***The effect of competing proteins on the nuclear import of v-ErbA in Xenopus oocytes.***

A. Oocytes were microinjected with 100 picograms of [^{35}S]-methionine labelled v-ErbA and histone H1 (to an intracellular concentration of 750 μM or 900 μM). Five to six hours after incubation at 18-20°C, protein was isolated from oocytes cytoplasmic (**C**) and nuclear (**N**) fractions, and analysed as described in Figure 3.1. (**LH1** or **[low]**: low histone H1 concentration of 750 μM; **HH1** or **[high]**: high histone H1 concentration of 900 μM)

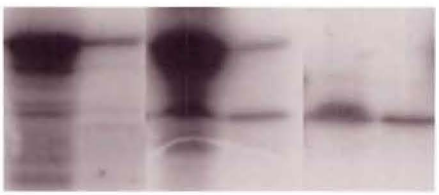
B. Oocytes were microinjected with 100 picograms of [^{35}S]-methionine labelled v-ErbA and 100 picograms of [^{35}S]-methionine labelled ribosomal protein L5 and analysed as described in **A.**

C. The percentages of nuclear accumulation from replicate experiments were determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of replicate batches (**n**) of pooled oocytes, as described in Figure 3.1. Standard error of the means is indicated by the bars. (v-ErbA[20°C]: n=9; v-ErbA[LH1]: n=3; v-ErbA[HH1]: n=3; v-ErbA+L5: n=3; L5: n=4)

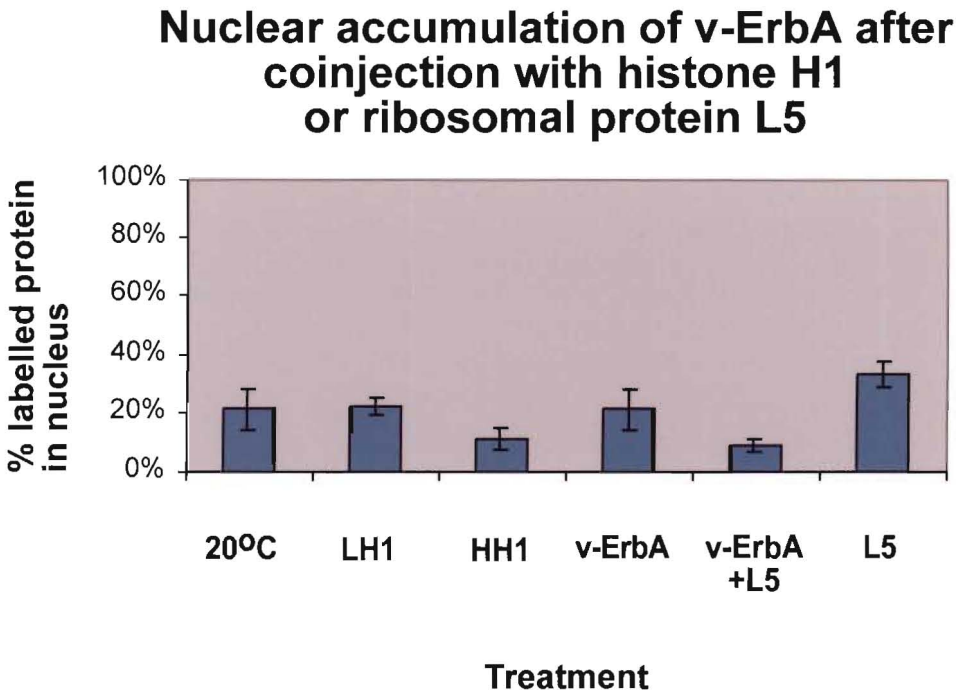
A


Histone H1
20°C [Low] [High]
C N C N C N

B


Coinjection
v-ErbA L5+ v-ErbA L5
C N C N C N

C


Nuclear accumulation of v-ErbA after coinjection with histone H1 or ribosomal protein L5
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
20°C
LH1
HH1
v-ErbA
v-ErbA +L5
L5
Treatment

Competition assays with L5 and individual NHRs, either TRα, TRβ, C122A, or v-ErbA, were performed. Data obtained from replicate experiments are displayed in Table 13. An inhibitory concentration of ribosomal L5 was determined by competing equimolar amounts of [^{35}S]-methionine-labelled L5 and unlabelled L5 (Figure 3.6C). Nuclear import of labelled L5 was inhibited ($p<0.001$) by equimolar amounts of unlabelled L5 as is demonstrated clearly in Figure 3.6 (B and D).

Coinjection with L5 did not lead to an alteration in the ratio of nuclear to cytoplasmic distributed protein in the case of TRα (Figure 3.7B). The distributions of TRβ and C122A were also not observed to have been affected by coinjection of L5 (Figures 3.8B and 3.9B). These findings were confirmed by Student's t-tests ($p>0.1$; as assessed from the replicate experiments displayed in Figures 3.7C, 3.8C, and 3.9C). The lack of competition observed with L5 by TRα, TRβ, and C122A suggests that independent mechanisms of transport are in operation for L5 as opposed to these NHRs. The import of v-ErbA was inhibited by coinjection with ribosomal protein L5 ($p=0.02$), with a reduction of nuclear localised protein from 21% to 9% (Figure 3.10, B and C). The observations made in these ribosomal protein L5 coinjection studies support the observations made in the histone H1 assays.

3.3.5 WGA and apyrase do not inhibit TR import in Xenopus oocytes

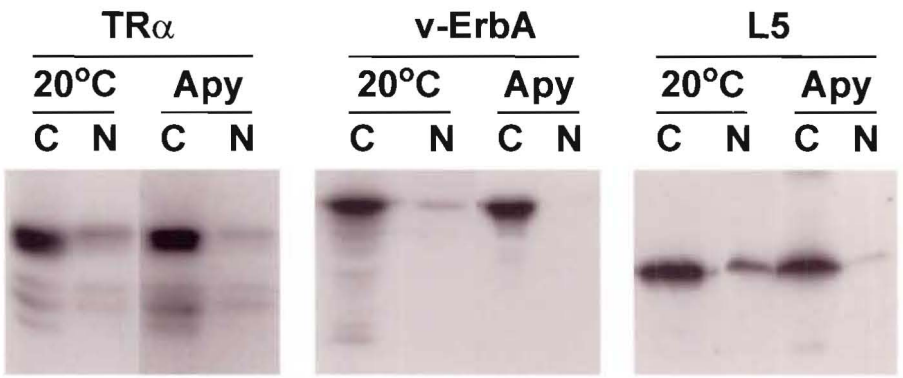
The two stages of transport can be separated by depletion of energy, or by chilling of the transport system to 4°C (Richardson *et al.,* 1988; Newmeyer and Forbes, 1988). ATP can be depleted by the addition of hydrolysing agents to test whether transport occurs after ATP depletion. Whereas ATP hydrolysis arrests transport at the docking stage, the second stage of transport, or translocation, can be inhibited by a variety of different agents; for example, addition of WGA (reviewed in Davis, 1995). WGA-induced inhibition of nucleocytoplasmic transport has been shown to be specific for facilitated transport processes (Dabauvalle *et al.,* 1988).

Figure 3.11. ***The effect of ATP depletion on the nuclear import of TRα, v-ErbA, and L5 in <u>Xenopus</u> oocytes.***

A. The effect of ATP depletion on the nuclear import of TRα, v-ErbA, and L5 was assessed by injection of oocytes with apyrase (to an intracellular concentration of 100 U/ml). After incubation for 30 minutes at 18-20°C, [^{35}S]-methionine labelled protein was injected into the cytoplasm, prior to incubation at 18-20°C for a further five to six hours. Nuclear import was assessed as described in Figure 3.1. (**C**: cytoplasmic fractions; **N**: nuclear fractions; **Apy**: Apyrase).

B. The percentages of nuclear accumulation from replicate experiments were determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of replicate batches (**n**) of pooled oocytes. Standard error of the means is indicated by the bars. (TRα[20°C]: n=8; TRα[Apy]: n=2; v-ErbA[20°C]: n=9; v-ErbA[Apy]: n=3; L5[20°C]: n=4; L5[Apy]: n=2)

A


TRα
v-ErbA
L5
20°C
Apy
20°C
Apy
20°C
Apy
C N C N
C N C N
C N C N

B

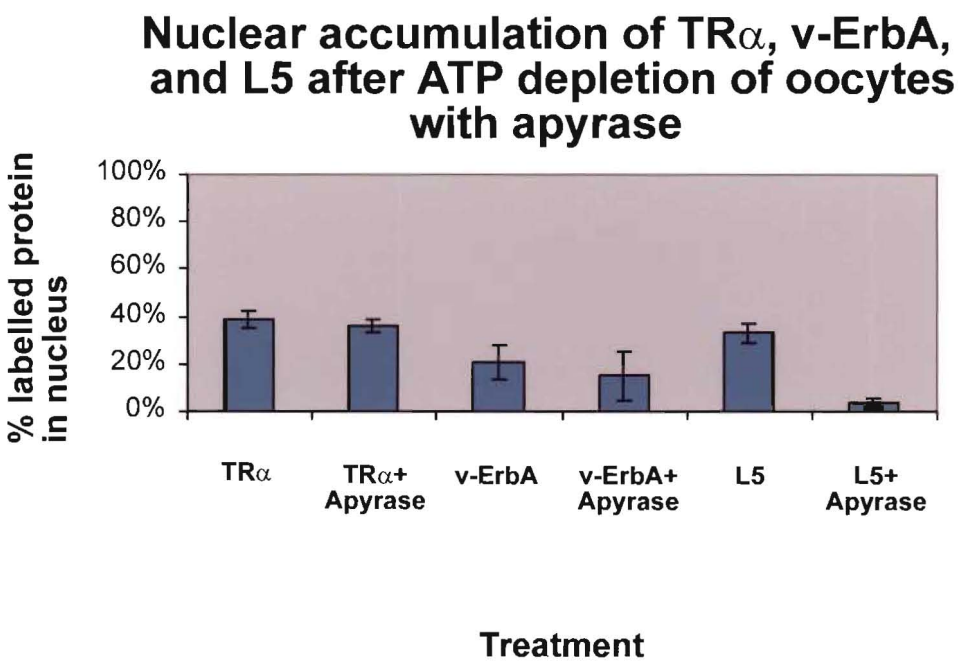
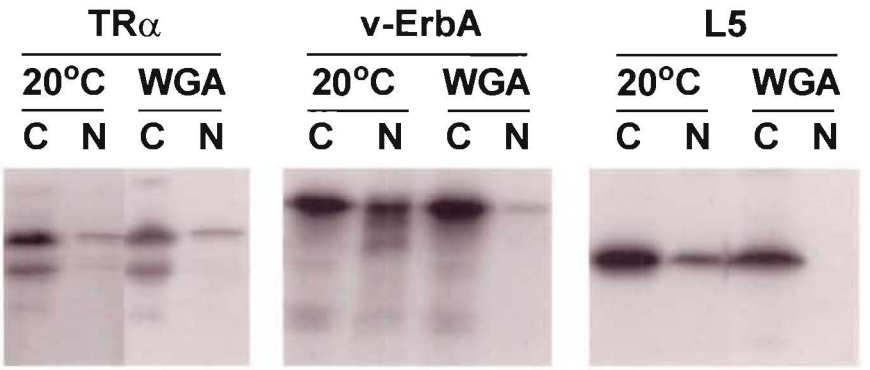

Nuclear accumulation of TRα, v-ErbA, and L5 after ATP depletion of oocytes with apyrase
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
TRα
TRα+ Apyrase
v-ErbA
v-ErbA+ Apyrase
L5
L5+ Apyrase
Treatment

Figure 3.12. ***The effect of WGA on the nuclear import of TRα, v-ErbA, and L5 in <u>Xenopus</u> oocytes.***

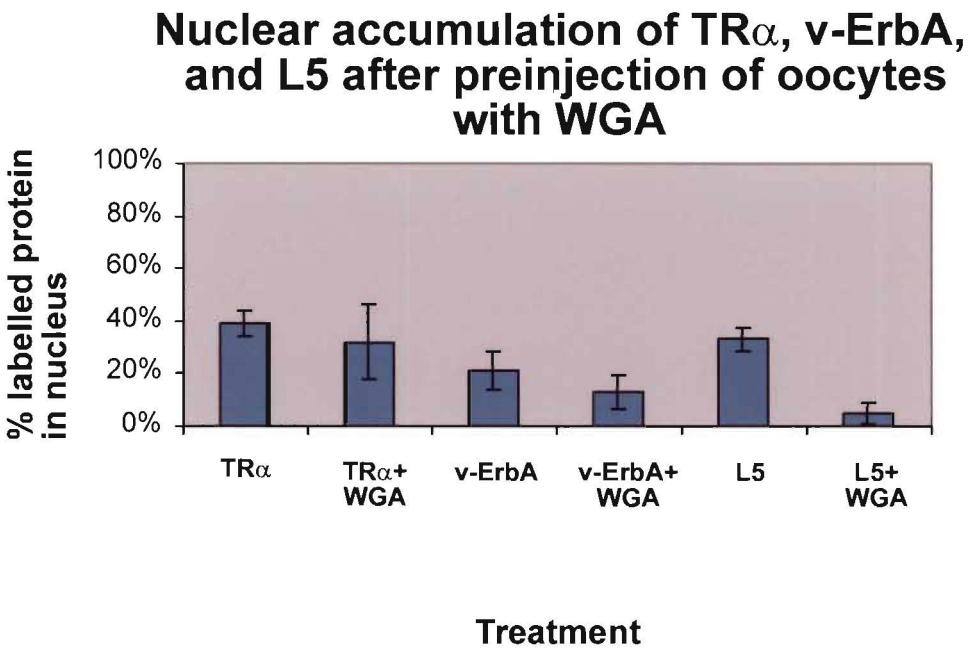
A. Oocytes were preinjected with WGA, at an intracellular concentration of 0.5 mg/ml, three hours prior to injection with [^{35}S]-labelled TRα, v-ErbA, or C122A. Five to six hours post-injection, oocytes were processed and analysed, as described in Figure 3.1. (**C**: cytoplasmic fractions; **N**: nuclear fractions; **WGA**: wheat germ agglutinin).

B. The percentages of nuclear accumulation from replicate experiments were determined by densitometry of the autoradiograms. Each graphed data point reflects the mean of replicate samples (**n**) of pooled oocytes. Standard error of the means is indicated by the bars. (TRα[20°C]: n=8; TRα[WGA]: n=3; v-ErbA[20°C]: n=9; v-ErbA[WGA]: n=3; L5[20°C]: n=4; L5[WGA]: n=2)

A


TRα
v-ErbA
L5
20°C
WGA
20°C
WGA
20°C
WGA
C N C N
C N C N
C N C N

B


Nuclear accumulation of TRα, v-ErbA, and L5 after preinjection of oocytes with WGA
% labelled protein in nucleus
100%
80%
60%
40%
20%
0%
TRα
TRα+ WGA
v-ErbA
v-ErbA+ WGA
L5
L5+ WGA
Treatment

Injections of apyrase and WGA are harsh treatments that may affect the integrity of the oocytes (personal observation). Treated oocytes are susceptible to disruption of the cell membranes and nuclear membranes during handling. In addition, the consistency of the cytoplasmic compartment may become either 'paste-like' or 'crumbly' in appearance, after microinjection and subsequent incubation. Therefore, all oocytes were carefully selected for use in these studies, and those with unstable membranes were discarded. Consequently, fewer oocytes could be obtained and in cases where less than two batches were processed, results are not presented (specifically for TRβ and C122A). Actual sample numbers are indicated in the legends of Figures 3.11 and 3.12.

Ribosomal protein L5 import was inhibited by both apyrase ($p<0.001$) and WGA ($p<0.001$), as expected for a protein transported through a facilitated process (Figures 3.11 and 3.12, respectively). WGA inhibition reduced L5 import from 33% to 5%, with a similar reduction to 4% observed for energy depletion assays. A passive diffusion mechanism of TRα import is supported by the results presented in Figures 3.11 and 3.12 (A and B). Import was not shown to be significantly inhibited by either ATP depletion ($p>0.1$) or WGA preinjection ($p>0.1$). Import of v-ErbA was also not shown to be inhibited by ATP depletion (15% nuclear; $p>0.1$) and WGA (13% nuclear; $p>0.1$), as shown in Figures 3.11 and 3.12 (A and B). Although two batches of v-ErbA-injected oocytes, for each treatment, were inhibited by ATP depletion and WGA preinjection (from the same two frogs for both treatments), a third batch was not.

3.3.6 Chilling does not affect EGFP-tagged TRα or C122A nuclear localisation in cultured NIH 3T3 cells.

Transport mechanisms for TR have not been characterised previously as, in the past, it has been generally accepted that the receptor population is constitutively nuclear (Perlman *et al.,* 1982; Puymirat *et al.,* 1989; Macchia *et al.,* 1992; Lee and Mahdavi, 1993). Experimentally, chilling has been shown to inhibit facilitated nuclear import of karyophilic proteins (Section 3.1.5). Lowering the incubation temperature of the cellular environment

during transport decreases the rates of enzyme-mediated processes. Pre-treatment of cultured cells with cycloheximide prevents *de novo* protein synthesis. Therefore, EGFP-tagged receptor molecules would have sufficient time to be localised to the correct cellular compartment. Possible outcomes for low temperature incubations and ATP depletion studies, classified by transport mechanisms, are displayed in Table 12. The data obtained from these investigations are summarised in Table 14.

Table 12 - Predicted outcomes for low temperature incubations and ATP depletion studies

Receptor	*Import mechanism*	*Export mechanism*	*Expected localisation*
TRα::EGFP	Passive diffusion	Passive diffusion	Nuclear (no change from physiological conditions)
	Passive diffusion	Facilitated transport	Nuclear (no change from physiological conditions)
	Facilitated transport	Passive diffusion	Cytoplasmic plus residual nuclear subpopulation
	Facilitated transport	Facilitated transport	Nuclear (no change from physiological conditions)
C122A::EGFP	Passive diffusion	Passive diffusion	Cytoplasmic (no change from physiological conditions)
	Passive diffusion	Facilitated transport	Cytoplasmic (perhaps some nuclear)
	Facilitated transport	Passive diffusion	Cytoplasmic (no change from physiological conditions)
	Facilitated transport	Facilitated transport	Cytoplasmic (no change from physiological conditions)

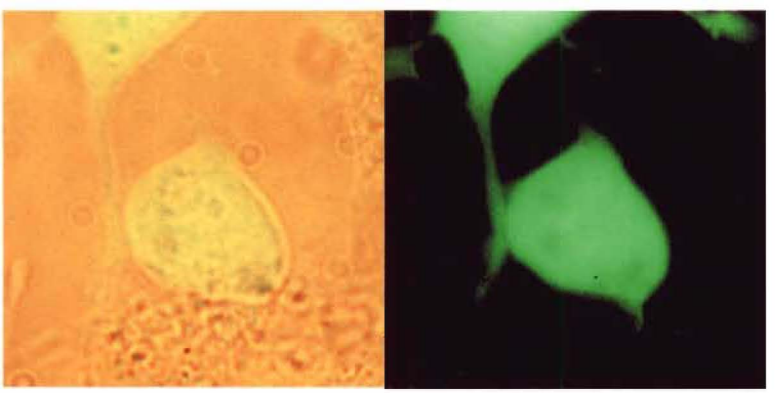
TRα and C122A tagged with EGFP were transfected into cultured cells that were exposed to low temperatures 48 hours post-transfection. Transfection efficiencies are recorded in Table 14. Neither TRα::EGFP nor C122A::EGFP were observed to significantly alter their distributions at 0-4°C from those observed in control incubations (Figure 3.13 compared with Figure 2.4), suggesting that any inhibition of TR import was not detectable and that export of receptor by passive diffusion had not occurred.

Figure 3.13. ***The effect of chilling on TRα::EGFP and C122A::EGFP localisation in transfected mammalian cells.***

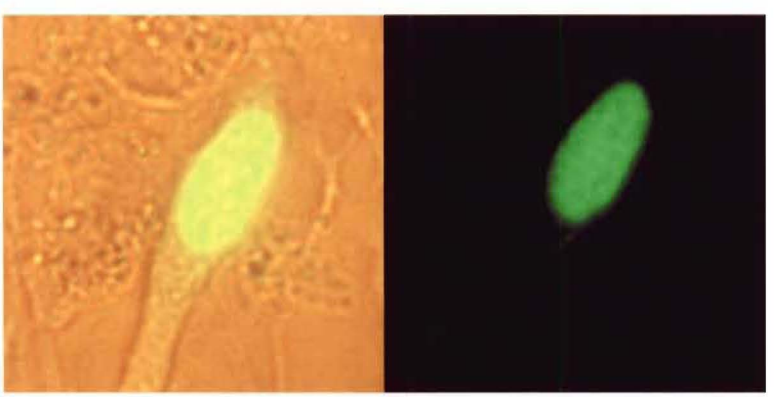
Plasmid constructs containing TRα or C122A fused to EGFP were transfected into NIH 3T3 cells, cultured to 50% confluence. Transfection medium was replaced by complete medium after 24 hours incubation at 37°C and 5% CO_2:95% air. After a further 24 hour incubation, cells were washed with D-PBS, and incubated in medium containing 50 μg/ml cycloheximide for thirty minutes, followed by three hours incubation at 0-4°C. Living cells were rinsed with D-PBS prior to visualisation with a fluorescent microscope, under a combination of bright-field and epifluorescence, as well as with epifluorescence only (as shown). Control transfections with EGFP were performed.

0-4°C incubations

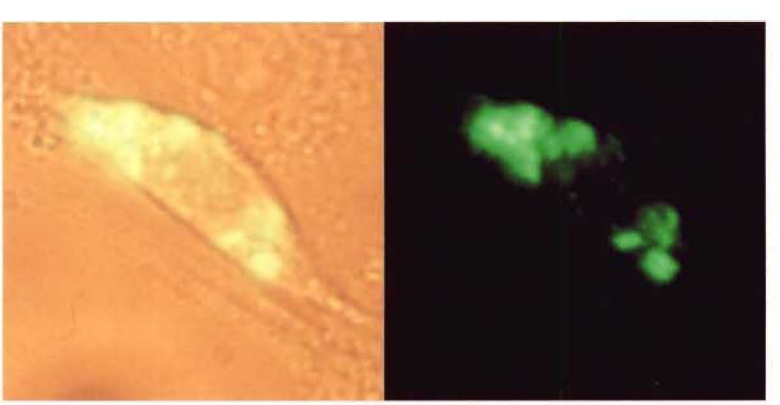
pEGFP



TRα



C122A



3.3.7 ATP depletion does not affect EGFP-tagged TRα and C122A nuclear localisation in cultured NIH 3T3 cells

A requirement for energy can be used to distinguish facilitated transport from passive diffusion (Newmeyer and Forbes, 1988; Richardson *et al.,* 1988; Akey and Goldfarb, 1989). Although the requirement is not direct, ATP can be depleted by the addition of hydrolysing agents to test whether transport occurs after ATP depletion (Section 3.1.4). A combination of 2-deoxyglucose and oligomycin in glucose minus DMEM were used to provide an ATP depleted medium for incubation of transfected cells. Possible outcomes for these experiments are summarised in Table 12.

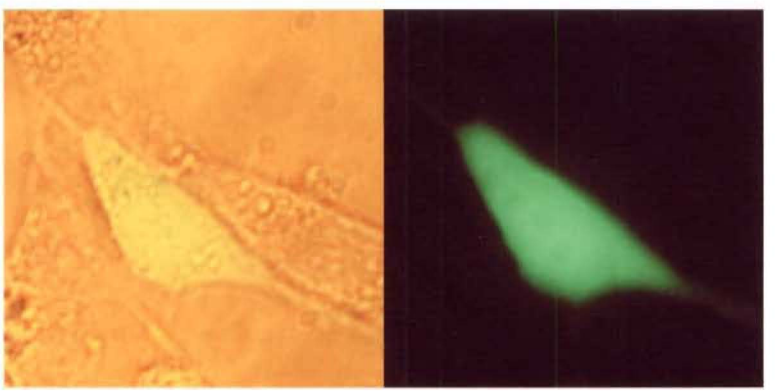
The distributions observed during low temperature incubations were mirrored by the results of energy depletion experiments in cultured NIH 3T3 cells (Figure 3.14 and Table 14). No significant shift in cellular distribution was observed for TRα::EGFP nor for C122A::EGFP, with the same implications as suggested for low temperature incubations in the previous section (3.3.6), that is, either import of receptor was not inhibited or any inhibition occurring was not at a detectable level.

Figure 3.14. ***The effect of ATP depletion on TRα::EGFP and C122A::EGFP localisation in transfected mammalian cells.***

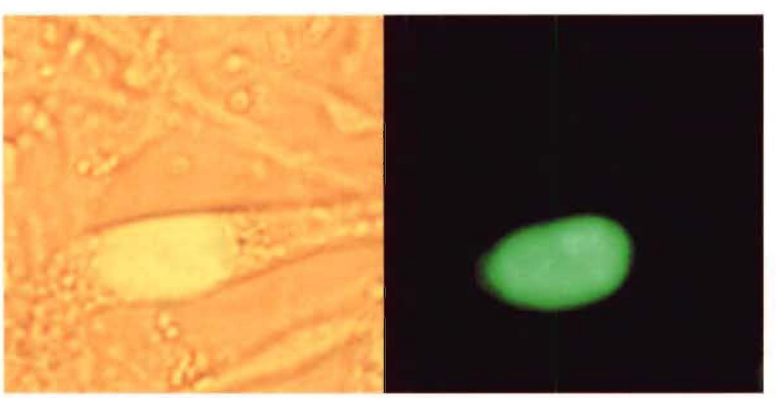
Cultured NIH 3T3 cells were transfected and incubated, as described in Figure 3.13 Twenty four hours after the culture medium replaced the transfection medium, cells were washed with D-PBS. Cells were incubated in medium containing 50 μg/ml cycloheximide for thirty minutes, followed by four hours incubation in cycloheximide-containing medium supplemented with 6 mM 2-deoxyglucose and 50 μM oligomycin. Cells were washed with D-PBS and viewed with a fluorescent microscope. Transfected cells are shown with a combination of bright-field and epifluorescence, as well as with epifluorescence only.

ATP depleted incubations

pEGFP



TRα



C122A

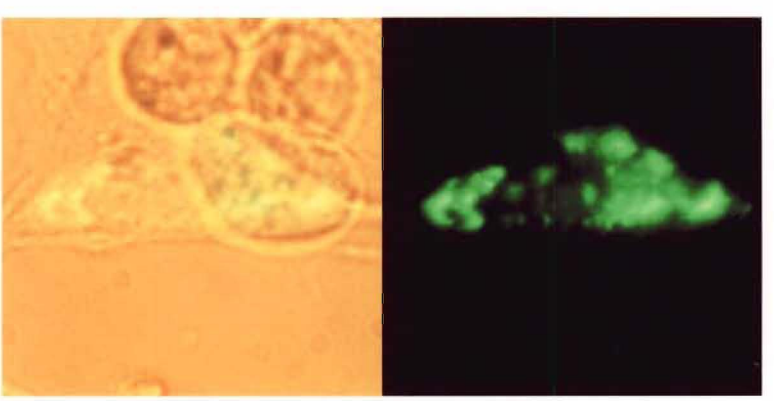


Table 13 - Summary of nucleocytoplasmic transport studies in *Xenopus* oocytes.

Receptor	*Direction of transport*	*Treatment*	*% nuclear protein*	*Student's t-test* [a]
TRα	import	Physiological conditions	39% ± 5%	reference
TRα	import	Chilling	40% ± 1%	NS
TRα	import	0°C+20°C	40% ± 8%	NS
TRα	import	WGA	32% ± 14%	NS
TRα	import	Apyrase	36% ± 3%	NS
TRα	import	Competition/low histone H1	41% ± 2%	NS
TRα	import	Competition/high histone H1	40% ± 4%	NS
TRα	import	Competition/L5	40% ± 3%	NS
TRα	export	Physiological conditions	62% ± 7%	reference
TRα	export	Chilling	86% ± 9%	p<0.001
TRβ	import	Physiological conditions	39% ± 3%	reference
TRβ	import	Chilling	38% ± 7%	NS
TRβ	import	0°C+20°C	37% ± 5%	NS
TRβ	import	Competition/low histone H1	43% ± 6%	NS
TRβ	import	Competition/high histone H1	42% ± 6%	NS
TRβ	import	Competition/L5	41% ± 2%	NS
TRβ	export	Physiological conditions	74% ± 3%	reference
TRβ	export	Chilling	87% ± 6%	p=0.03
C122A	import	Physiological conditions	41% ± 2%	reference
C122A	import	Chilling	40% ± 5%	NS
C122A	import	0°C+20°C	41% ± 9%	NS
C122A	import	Competition/low histone H1	38% ± 5%	NS
C122A	import	Competition/high histone H1	37% ± 7%	NS
C122A	import	Competition/L5	38% ± 5%	NS
C122A	export	Physiological conditions	72% ± 6%	reference
C122A	export	Chilling	87% ± 6%	p=0.06
v-ErbA	import	Physiological conditions	21% ± 7%	reference
v-ErbA	import	Chilling	25% ± 8%	NS
v-ErbA	import	0°C+20°C	22% ± 2%	NS
v-ErbA	import	WGA	13% ± 7%	NS
v-ErbA	import	Apyrase	15% ± 10%	NS
v-ErbA	import	Competition/low histone H1	22% ± 3%	NS
v-ErbA	import	Competition/high histone H1	11% ± 3%	p=0.05
v-ErbA	import	Competition/L5	9% ± 2%	p=0.02

v-ErbA	export	Physiological conditions	87% ± 5%	reference
v-ErbA	export	Chilling	82% ± 2%	NS
L5	import	Physiological conditions	33% ± 4%	reference
L5	import	Chilling	18% ± 2%	p<0.001
L5	import	0°C+20°C	29% ± 4%	NS
L5	import	WGA	5% ± 4%	p<0.001
L5	import	Apyrase	4% ± 1%	p<0.001
L5	import	Competition/low histone H1	21% ± 2%	p<0.001
L5	import	Competition/high histone H1	16% ± 2%	p<0.001
L5	import	Competition/L5	8% ± 1%	p<0.001
L5	export	Physiological conditions	68% ± 7%	reference
L5	export	Chilling	95% ± 5%	p<0.001

[a] Student's t-test values are expressed as the probability that the experimental treatments were significantly different at p<0.1. Treatments were compared with the appropriate values obtained in oocytes incubated at physiological conditions. (**NS**: not significant)

Table 14 - Summary of nucleocytoplasmic transport studies in NIH 3T3 cells.

Receptor	*Sample size (n)*	*Treatment*	*Distribution of protein*	*Transfection efficiency*
TRα::EGFP	500	physiological conditions	100% nuclear	21% ± 6%
TRα::EGFP	384	0°C-4°C	99.48% nuclear	14% ± 7%
TRα::EGFP	500	ATP minus	99.25% nuclear	13% ± 7%
C122A::EGFP	297	physiological conditions	100% cytoplasmic	11% ± 5%
C122A::EGFP	254	0°C-4°C	100% cytoplasmic	8% ± 5%
C122A::EGFP	261	ATP minus	100% cytoplasmic	9% ± 3%
EGFP	500	physiological conditions	100% (diffused through entire cell)	71% ± 13%
EGFP	500	0°C-4°C	100% (diffused through entire cell)	66% ± 16%
EGFP	500	ATP minus	100% (diffused through entire cell)	64% ± 16%

3.4 Discussion

The results presented in this chapter demonstrate that the nuclear import of TRα, TRβ, and DNA-binding mutant C122A through the NPC in *Xenopus* oocytes occurs via passive diffusion (Section 3.4.1). Import is temperature-independent, is not sensitive to ATP depletion or WGA, and is not competed by histone H1 or ribosomal protein L5. However, nuclear import of v-ErbA is via a facilitated mechanism. In contrast, efflux of TRα, TRβ, and C122A molecules from the nuclear compartment is temperature dependent (Section 3.4.3).

In Chapter Two, it was demonstrated that DNA binding was not a requirement for nuclear retention of TR. It also appears that a loss of integrity in the DNA binding domain does not influence the transport mechanisms operating in *Xenopus* oocytes (Section 3.4.1).

In addition, low temperature incubations and ATP depletion assays were performed in TRα::EGFP and C122A::EGFP transfected NIH 3T3 cells, to begin to characterise transport mechanisms and to determine whether nucleocytoplasmic shuttling of receptor occurs. No change in receptor localisation was observed. The implications of this observation are discussed in Section 3.4.4.

3.4.1 Nuclear import of TRα, TRβ, and C122A in Xenopus oocytes occurs by a passive process

The mechanism of nuclear import of a protein can be characterised by a range of assays, including low temperature incubations, energy depletion, WGA inhibition, and competition assays. Categorisation of protein transport into facilitated or passive processes cannot be made simply upon size as several proteins that are smaller than the limit for diffusion are transported by a facilitated mechanism; such as calmodulin (Pruschy *et al.*, 1994) and histone H1 (Breeuwer and Goldfarb, 1990; Kurz *et al.*, 1997). TRα, TRβ, and C122A fall within the upper size limits for diffusion through the NPC (46.8 kDa, 52 kDa, and 52 kDa, respectively), whilst v-ErbA exceeds the limit (75 kDa).

As TR has been reported to be constitutively localised to the nuclei of cultured somatic cells (Perlman *et al.,* 1982; Kumara-Siri *et al.,* 1986; Horowitz *et al.,* 1989; Puymirat *et al.,* 1989; Macchia *et al.,* 1992; Lee and Mahdavi, 1993), nucleocytoplasmic transport mechanisms had not been previously investigated. Since nuclear localisation of TRα, TRβ, and C122A is not constitutive in *Xenopus* oocytes (approximately 40% is nuclear), as determined by the investigations presented in this thesis, these oocytes provide a unique model cell system for defining these transport mechanisms. An array of assays was performed to determine the mechanisms of transport for TRα, TRβ, DNA-binding mutant C122A, and oncoprotein v-ErbA.

Import of the TR isoforms and C122A in *Xenopus* oocytes occurred to the same extent for all three receptors in a temperature independent manner, consistent with passive diffusion. This finding provides further evidence that nuclear accumulation of TR is due to nuclear retention of receptor molecules by specific binding to nuclear retention sites (refer Section 2.4.2). In support of a passive import mechanism, nuclear import of the receptors was not inhibited by competition with facilitated import of histone H1 or L5. Active and facilitated transport of proteins are saturable processes and as such, they are sensitive to competition by other substrates that follow the same pathway; for example, histone H1 (Breeuwer and Goldfarb, 1990; Harootunian *et al.,* 1993; Kurz *et al.,* 1997). Further, the effects of energy depletion and WGA inhibition on TRα and v-ErbA import were assessed in *Xenopus* oocytes. WGA blocks the uptake of a variety of karyophilic proteins independent of their molecular weight and inhibition of transport has been shown to be specific for facilitated transport processes (Dabauvalle *et al.,* 1988). Facilitated transport can be described more completely as an energy dependent process in most cases, without which transport is arrested at the docking stage (Newmeyer and Forbes, 1988; Richardson *et al.,* 1988; Akey and Goldfarb, 1989). Neither apyrase nor WGA treatments inhibited TRα import to the extent that L5 was inhibited. This finding might suggest that two import pathways exist for TRα in *Xenopus* oocytes, and that whilst passive diffusion of TRα is not inhibited, secondary import by a facilitated mechanism is inhibited. In addition, a high degree of variability between oocyte batches biased the statistical t-tests performed. This bias could be countered by an increase in replicate number, although further experiments could not be performed in the time frame of this thesis. Therefore, whilst it appears that most nuclear proteins, including proteins of a size

suitable for diffusion (upper limit 40 to 60 kDa), are transported by a facilitated mechanism requiring the interaction with transport complexes, the influx of TRα, TRβ, and C122A into the *Xenopus* oocyte occurs by passive diffusion and not by a facilitated mechanism.

In contrast to the results observed for TR import, the mechanism of v-ErbA import in *Xenopus* oocytes is not passive diffusion. Although, v-ErbA import was not significantly inhibited in comparison to L5 by low temperature incubations, WGA, or ATP depletion, import was significantly affected by coinjection with L5 and high levels of histone H1. Oocytes injected with v-ErbA were more likely to display deterioration of the nuclear membrane after incubation at low temperatures. A loss of membrane integrity, previously described to be induced by v-ErbA (Nagl *et al.*, 1997), would lead to 'leakiness' of the nuclear compartment, although this occurred over a longer time frame. In addition, it must be re-emphasised that the statistical t-tests performed to compare treatments are influenced by high variability between samples and low replicate numbers of samples.

A potential difficulty in assessing v-ErbA import was identified during the course of this study. As discussed in Section 2.4.5, the majority of cytoplasmically microinjected v-ErbA may have been sequestered in the cytoplasmic compartment in association with hsp90, in a form that was not competent for nuclear import.

3.4.2 Role of the TR NLS in nuclear localisation

Small proteins lacking an NLS diffuse through the central channel of the NPC regardless of temperature (Breeuwer and Goldfarb, 1990). In light of the passive mechanism observed for nuclear import of TRα, TRβ, and C122A, it is important to note that NLSs have been identified for TR. Dang and Lee (1989) identified an NLS in the DBD of chicken TR, and similarly an NLS was identified in the hinge regions of both rat $TR\alpha_1$ (Lee and Mahdavi, 1993) and human $TR\beta_1$ (Zhu *et al.*, 1998). Therefore, it would seem that these NLSs would be redundant with a simple passive import process. However, it must be pointed out that presence of an NLS may not necessitate import via a facilitated mechanism. Although the 41 kDa catalytic subunit of cAMP-dependent protein kinase has lysine and arginine residues

that could comprise an NLS, it was concluded that diffusion was sufficient to explain most aspects of the subunit's transport (Harootunian *et al.*, 1993). It is possible that subtle regulation of TR function could occur by import of a small population of receptor by a facilitated mechanism, which might show more rapid kinetics in comparison to the relatively slow process of passive diffusion. Signal-mediated import could ensure targeting of TR molecules to specific association sites with the oocyte nucleus, rather than delivery en masse of the majority of nuclear receptor by undirected passive diffusion. Also to be considered is the manner in which the NLSs were isolated. Due to the close proximity of the NLSs to the DBD of the receptor (Dang and Lee, 1989; Lee and Mahdavi, 1993; Zhu *et al.*, 1998), isolation of this function may be due to disruption of another function, perhaps even mutation of an NES (Laskey and Dingwall, 1993; Schmidt-Zachmann *et al.*, 1993). However, in the case of PR, a specific NES (distinct from an NLS) may not be required for PR export, and export may be mediated through an NLS (Guiochon-Mantel *et al.*, 1994). Evidence for a signal-mediated mechanism of transport is provided by the temperature dependence of TRα, TRβ, and C122A export. It remains to be determined whether export is mediated by the NLS, or by a specific NES.

3.4.3 Export of TR isoforms, C122A, and v-ErbA is temperature dependent in Xenopus oocytes.

Localisation of TR, v-ErbA, and C122A has been demonstrated not to be constitutively nuclear in oocytes (Section 2.3.2). As a result of this observation, TR and variants were introduced into oocyte nuclei to determine whether receptor was localised in the nucleus or whether nuclear export occurred. Nuclear export of several NHRs has been shown to occur; including, GR, AR, and MR (Yang and DeFranco, 1994), PR (Guiochon-Mantel., 1991), and ER (Dauvois *et al.*, 1993).

The presence of TR export in oocytes, in combination with passive diffusion of receptor into the nucleus, is indicative of nucleocytoplasmic shuttling. Nucleocytoplasmic shuttling has been demonstrated in the NHR superfamily. Liganded PR and GR shuttling between nuclei of heterokaryon fusions was identified (Guiochon-Mantel *et al.*, 1991; Madan

and DeFranco, 1993; respectively). Heterokaryon experiments performed with the TRα::EGFP clone used in the study described in this thesis, carried out in Dr L. A. Allison's laboratory (College of William and Mary), have suggested shuttling of receptor (personal communication). It has been proposed that nucleocytoplasmic shuttling of proteins could be implicated in two types of protein regulation (Nigg, 1990; Nigg, 1997). Firstly, shuttling proteins may act as carrier molecules for nuclear import or export of other proteins or RNA. For example, it has been proposed that the yeast protein Gle1p acts as an essential mRNA export factor (Murphy and Wente, 1996). Secondly, cytoplasmic regulation of nuclear activities may be regulated by transient exposure of the shuttling protein to the cytoplasmic compartment. This cytoplasmic exposure would allow modification of the receptor (by phosphorylation or other means) resulting in altered affinities for specific nuclear protein interactions. IκB inhibits nuclear localisation of NFκB/Rel transcription factors through interactions formed in the cytoplasmic compartment (Baeuerle and Henkel, 1994). Inactivation of IκB leads to nuclear import of the NFκB/Rel transcription factors. IκB appears to contain an NES and it has been suggesting that shuttling of the inhibitor may play a role in terminating NFκB signal transduction through a negative feedback mechanism (Fritz and Green, 1996). The significance of nucleocytoplasmic shuttling for TR seems most likely to be involved with cytoplasmic regulation. Indeed, as detailed in Section 2.4.1, phosphorylation of nuclear receptor, but not cytoplasmic TR, has been observed (L. A. Allison, personal communication). Regulation of shuttling TR in the cytoplasmic compartment would therefore provide a means to transfer information from this compartment to the nucleus.

3.4.4 No effect of chilling or ATP depletion on localisation of EGFP-tagged TRα or C122A is observed in cultured cells

Low temperature incubations and energy (ATP) depletion have been demonstrated to inhibit the facilitated nuclear transport of a variety of proteins, including histone H1 import in PtK1 cells (Breeuwer and Goldfarb, 1990; Kurz *et al.*, 1997); nucleocytoplasmic transport of GR, AR and MR (Yang and DeFranco, 1994); import of PR (Guiochon-Mantel *et al.*, 1994) and nucleoplasmin in tissue culture cells (Richardson *et al.*, 1988).

No difference in distribution of either TRα::EGFP or C122A::EGFP was observed in chilled versus warm cells, or in energy-depleted versus control cells. The lack of observable difference could be accounted for a variety of reasons. Firstly, TRα::EGFP may diffuse into the nuclei of NIH 3T3 cells and then be retained, therefore chilling and ATP depletion would not inhibit the process. This seems unlikely as the EGFP-tagged protein would exceed the upper size limit for diffusion, and at 73.8 kDa is similar in size to v-ErbA. However, if this is the case, and if nucleocytoplasmic shuttling of TRα occurs, this implies that export of receptor is temperature and energy dependent, enabling TRα::EGFP to accumulate in the nucleus in association with binding sites. Secondly, import of TRα::EGFP may occur by a rapid facilitated mechanism, meaning that the 30 minute incubation period in cycloheximide would be sufficient for import of all receptor to occur prior to chilling. Any nucleocytoplasmic shuttling would be constrained as previously described. Alternatively, low levels of receptor present in the cytoplasmic compartment (retained or exported) might be undetectable by fluorescent microscopy. Therefore, the findings made for the low temperature incubations and energy depletion assays do not allow distinctions to made between, firstly, active versus passive import, and, secondly, inhibition of shuttling as opposed to absence of nuclear export. In order to assess the two events, import and export or shuttling, it would be necessary to establish whether export or shuttling occurs. Assessment of TRα export could be performed in heterokaryon fusion cells in experiments similar to those performed for PR (Guiochon-Mantel *et al.,* 1989; Guiochon-Mantel *et al.,* 1991) and GR (Madan and DeFranco, 1993). Experimentation is currently in progress in Dr L. A. Allison's laboratory (College of William and Mary). Export and import could also be assessed by the introduction of TRα::EGFP into one cell nucleus by microinjection and examination of the movement of receptor to the other nucleus. As a control for these experiments, a protein that is exported under chilled and energy-depleted conditions should be included. The inhibitory action of cycloheximide on de novo protein synthesis could also be assessed by introduction of a CAT expression vector and subsequent assay for synthesised protein by enzyme linked immunosorbant assay (ELISA).

In contrast to the nuclear distribution of TRα::EGFP, EGFP-tagged C122A is cytoplasmically distributed. In light of the absence of receptor detected in the nuclear compartment of both living cells (Section 2.3.3) and fractionated cells (Section 2.3.7), it

seems unlikely that import occurs. Therefore, it would be interesting to introduce C122A by microinjection into the nuclei of NIH 3T3 cells to observe whether the DNA-binding mutant is retained in the nucleus or exported to the cytoplasm, especially as C122A is exported in *Xenopus* oocytes (Section 3.3.2). It has been previously observed that when non-nuclear proteins are microinjected into the nucleus they are unable to be exported (Dingwall *et al.,* 1982; Lanford *et al.,* 1986; Mandell and Feldherr, 1990; Guiochon-Mantel *et al.,* 1991).

Chapter 4

Conclusion

The study presented in this thesis examines the distribution and nucleocytoplasmic transport of the α- and β-isoforms of TR, a DNA-binding mutant of TRβ (C122A), and the AEV-derived oncoprotein v-ErbA which no longer binds T_3. Results presented in Chapter Two demonstrate that distributions of TR and its variants, introduced into *Xenopus* oocytes, vary from those described in the literature for mammalian and avian cells. These observations were specific for the oocyte cell context. Incubation of oocytes in T_3 induced a significant shift of TRα, TRβ, and C122A to the oocyte nucleus indicating that, either, nuclear binding sites had become more accessible, or, that additional sites had become accessible. In contrast, TRα::EGFP was localised to the nuclei of cultured NIH 3T3 cells and incubation in the absence or presence of T_3 induced no changes in distribution. Examination of the nuclear transport of receptors revealed that TRα, TRβ, and C122A entered the oocyte nucleus by passive diffusion, whilst export occurred by a temperature dependent mechanism. There was some suggestion that nuclear import of a small subpopulation of TR might occur by a facilitated mechanism. Export of TR has not been described in the literature, and observation of TR export in oocytes suggests that nucleocytoplasmic shuttling of TR may occur. Integrity of the TR DBD was not essential for correct localisation or transport of receptor, as characterised by use of DNA-binding mutant C122A.

The results contained in Chapters Two and Three provide insight into interactions of TR and variants with the cellular transport machinery and nuclear retention sites in the nuclear matrix. Potential regulatory mechanisms for TR action have also been discussed in these two chapters. Therefore, Chapter Four is designed to place the implications of TR localisation and transport mechanisms in the context of regulation of TR activity, and with other members of the NHR superfamily.

4.1 Comparison of TR with other members of the NHR superfamily

As a member of the NHR superfamily, TR shares a common structure with other NHRs (Figure 1.1). Function is structurally related and, therefore, it is expected that receptors in the superfamily will have features in common.

The NHR superfamily displays a range of distributions within the cellular compartments. The classical model proposed that NHRs were cytoplasmic in the absence of ligand binding and were imported after binding in order to associate directly with DNA, activating or repressing genes by way of response elements (reviewed in Nigg, 1990). This model was being revised by 1986 as the presence of nuclear pools of unliganded receptor were described for ER and PR (King and Greene, 1984; Perrot-Applanat *et al.,* 1985).

Structural integrity of the DBD is an important determinant for the nuclear localisation and tight nuclear binding of GR (Sackey *et al.,* 1996). The study presented in this thesis demonstrates that maintenance of DNA binding is not the sole determinant for nuclear localisation and import of TR, as nuclear import of C122A (a DNA-binding mutant) proceeds at the rate and to the degree observed for wild type receptor in oocytes but not in cultured NIH 3T3 cells.

Association of NHRs with nuclear sites separate from DNA has also been described, with ER and GR localising to the nuclear matrix (Alexander *et al.,* 1987; van Steensel *et al.,* 1995), VDR localising to discrete nuclear foci (Barsony *et al.*, 1997), and GR and MR concentrating in approximately 1000 clusters within the nucleus (van Steensel *et al.*, 1996). Additionally, it was demonstrated that the distributions of several NHRs were dynamic rather than static in the absence of ligand; that is, receptors shuttled between the nuclear and cytoplasmic compartments (reviewed in Guiochon-Mantel *et al.,* 1996). Figure 4.1 summarises what is currently known about the trafficking of NHRs.

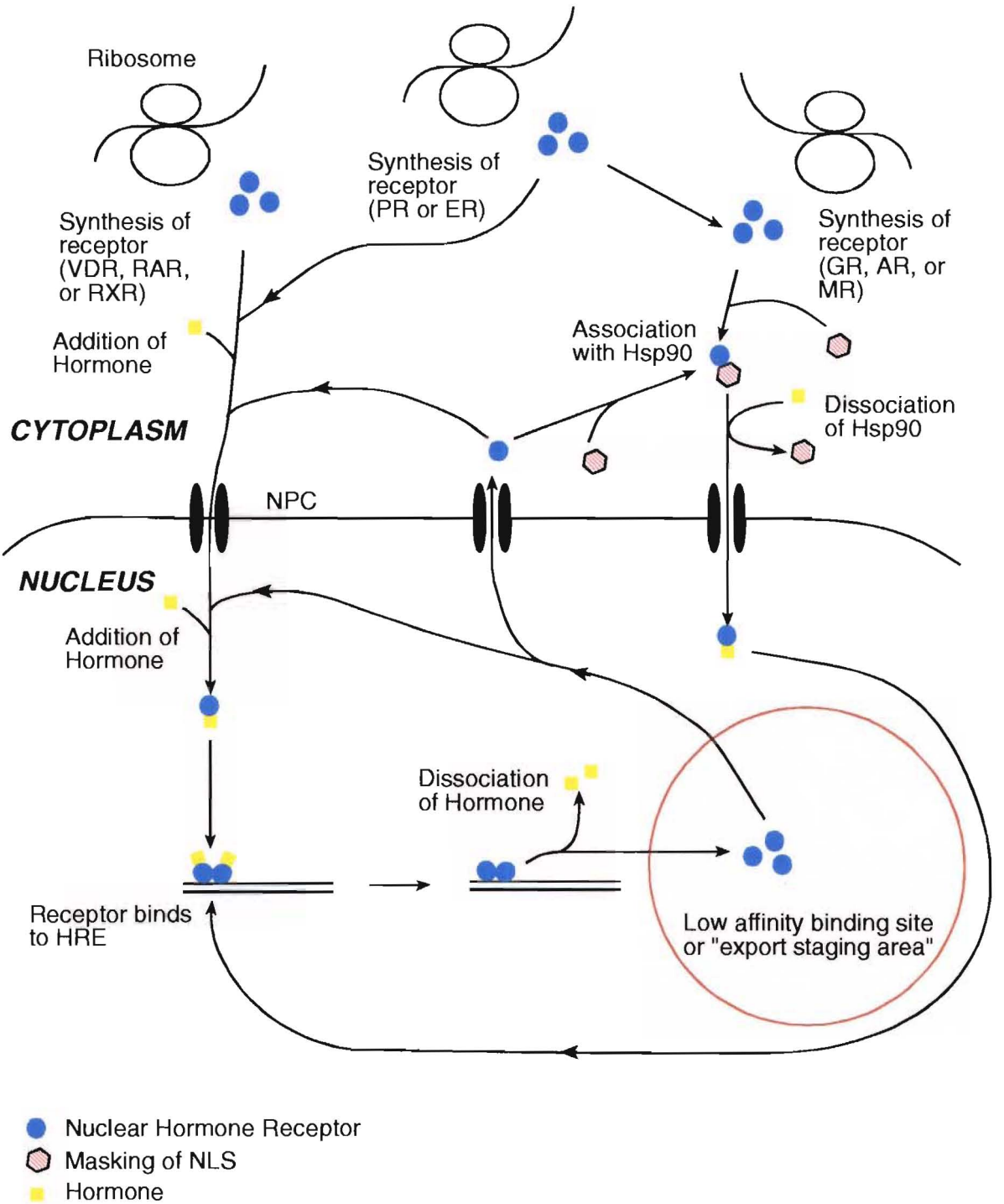


Figure 4.1 - Localisation of nuclear hormone receptors. ***The trafficking of various NHRs and alternative pathways for receptor localisation between the cytoplasmic and nuclear compartments are displayed. This figure is collated from previously reported studies discussed in the introductory sections contained in this thesis (Chapter 3).***

Some members of the NHR superfamily have been shown to complex with hsp90 in the cytoplasmic compartment (Feldherr and Akin, 1994; reviewed in Brasch and Ochs, 1995; Dittmar *et al.,* 1996), which effectively anchors the receptor and prevents nuclear import. In terms of hsp90 association, TR is similar to VDR, RAR, and RXR, as they all remain free from complex formation. However, unliganded TR is reported to be constitutively nuclear in cultured cells (for example, NIH 3T3 cells, as demonstrated in this study) and has been shown in this study to be distributed between the cytoplasmic and nuclear compartments in *Xenopus* oocytes. In this way, the constitutively nuclear distributions observed in cultured cells (Perlman *et al.,* 1982; Kumara-Siri *et al.,* 1986; Horowitz *et al.,* 1989; Puymirat *et al.,* 1989; Macchia *et al.,* 1992; Lee and Mahdavi, 1993) and in NIH 3T3 cells (in this study) are more similar to those observed for PR (Perrot-Applanat *et al.,* 1985) or ER (King and Greene, 1984) than for VDR (Barsony *et al.,* 1997), MR (Lombès *et al.,* 1990; Robertson *et al.,* 1993; Lombès *et al.,* 1994; Fejes-Tóth *et al.,* 1998), GR (Fuxe *et al.,* 1985; Wikström *et al.,* 1987; Rossini and Malaguti, 1994; Akner *et al.,* 1995), or AR (Simental *et al.,* 1991; Jenster *et al.,* 1993).

Perhaps the most surprising observation made during this study is that TR appears to passively diffuse into the nuclei of *Xenopus* oocytes, regardless of the presence of an NLS (Dang and Lee, 1989; Lee and Mahdavi, 1993; Zhu *et al.,* 1998), although there is the potential that two alternative pathways exist. In contrast to TR, import is an energy and temperature dependent, facilitated process for larger NHRs; such as, GR (94 kDa), AR (110 kDa), MR (107 kDa; Yang and DeFranco, 1994), and PR (90 kDa; Guiochon-Mantel *et al.,* 1994). Export of TR in *Xenopus* oocytes is by a temperature dependent mechanism suggesting a facilitated mechanism, which is different from the energy independent mechanism of PR export (Guiochon-Mantel *et al.,* 1991).

Hormone association is necessary for nuclear import of AR, GR, and MR, whereas dissociation of hormone results in dissociation of the receptor molecules from chromatin and subsequent nuclear export (Yang and DeFranco, 1994). Whilst ligand binding is not obligatory for import of TR in *Xenopus* oocytes, localisation of receptor to the nucleus is enhanced in the presence of hormone. Nuclear localisation of EGFP-tagged TRβ is also

enhanced in the presence of T_3, displaying a shift from 60% to 85% nuclear (Zhu *et al.*, 1998). However, as TR is constitutively localised to the nuclei of somatic cells (Perlman *et al.*, 1982; Kumara-Siri *et al.*, 1986; Horowitz *et al.*, 1989; Puymirat *et al.*, 1989; Macchia *et al.*, 1992; Lee and Mahdavi, 1993), and observed in this study in NIH 3T3 cells, ligand binding is not an absolute requirement for nuclear import. Ligand binding is also not obligatory for nuclear localisation of PR (Perrot-Applanat *et al.*, 1985) and ER (King and Greene, 1984; Welshons *et al.*, 1984; Welshons *et al.*, 1985), although binding to target DNA is necessary.

A population of TR introduced into *Xenopus* oocytes is mobile and able to be exported from the nucleus through the NPC. This suggests that TR has the ability to cycle through the nuclear and cytoplasmic compartments in oocytes in the manner that PR (Guiochon-Mantel *et al.*, 1991) and GR (Madan and DeFranco, 1993) have been shown to shuttle in heterokaryon fusion cells. Evidence of EGFP-tagged TR shuttling has also been observed in heterokaryon fusion cells (L. A. Allison, personal communication), suggesting a more universal mechanism.

4.2 Nuclear localisation of TR

This section describes a model formulated to account for the phenomena observed during the course of this study. Evidence for the interactions presented in this model is provided by the original investigations contained in this thesis placed in context with previous studies of TR. Figure 4.2 displays the possible interactions TR makes with cell components during its transport into, through, and out of the nuclear compartment.

The fate of TR is determined by the cellular context in which the receptor is placed or synthesised, although a universal model for TR localisation is proposed in Figure 4.2. TR is imported into the nuclei of cultured cells and remains there. Alternatively, TR associates with anchoring complexes in the cytoplasmic compartment (for which a case may be made in *Xenopus* oocytes). TR molecules in excess of anchor complexes would remain free to diffuse into the nucleus. Dissociation of the anchoring complex may be due directly to hormone

binding or it might be due to a phosphorylation event induced by hormone binding. Evidence for receptor phosphorylation was detailed in Section 2.4.1. Liganded TR diffuses into the nuclear compartment, or in the case of cultured cells, receptor binds ligand in the nucleus or during its passage through the cytoplasm. Alternatively, in oocytes, unliganded TR may be imported and be more likely to be retained if it binds T_3 in the nucleus.

Alternative associations can be formed between TR and the nuclear architecture. Three fates are described for TR. Firstly, TR binds TREs in the cell genome. Unliganded TR is also found in association with TREs in cultured cells. Secondly, TR associates with retention sites within the nucleus, such as the nuclear matrix, or with low affinity binding sites. The nuclear matrix is associated with actively transcribed genes and their transcription factors (Zeng *et al.*, 1997). Therefore, TR might trans-activate gene expression while associated with the nuclear matrix, which has positioned TR in close proximity to the chromatin allowing TRE binding by TR to occur (not shown in Figure 4.2). Lastly, shuttling of TR occurs with the efflux of receptor via a facilitated mechanism. Dissociation of hormone within the nuclear compartment is likely to result in a rearrangement of receptor associations, and may therefore induce shuttling of TR. This rearrangement may also be a movement of TR to quasifunctional sites or low affinity retention sites.

Several requirements must be met for the interactions proposed in this model to occur. DNA binding appears to be essential for nuclear localisation of receptor (EGFP-tagged) in NIH 3T3 cells but not necessarily in *Xenopus* oocytes. Association of TR with TREs is dependent on integrity of the DBD which is, however, not necessary for interactions with alternative sites in oocytes. The size of TR is also a determining factor in receptor transport as the viral fusion protein, v-ErbA, is too large to passively diffuse through the NPC. In addition, hsp90 complexes with v-ErbA, but not TR, acting as a cytoplasmic anchor.

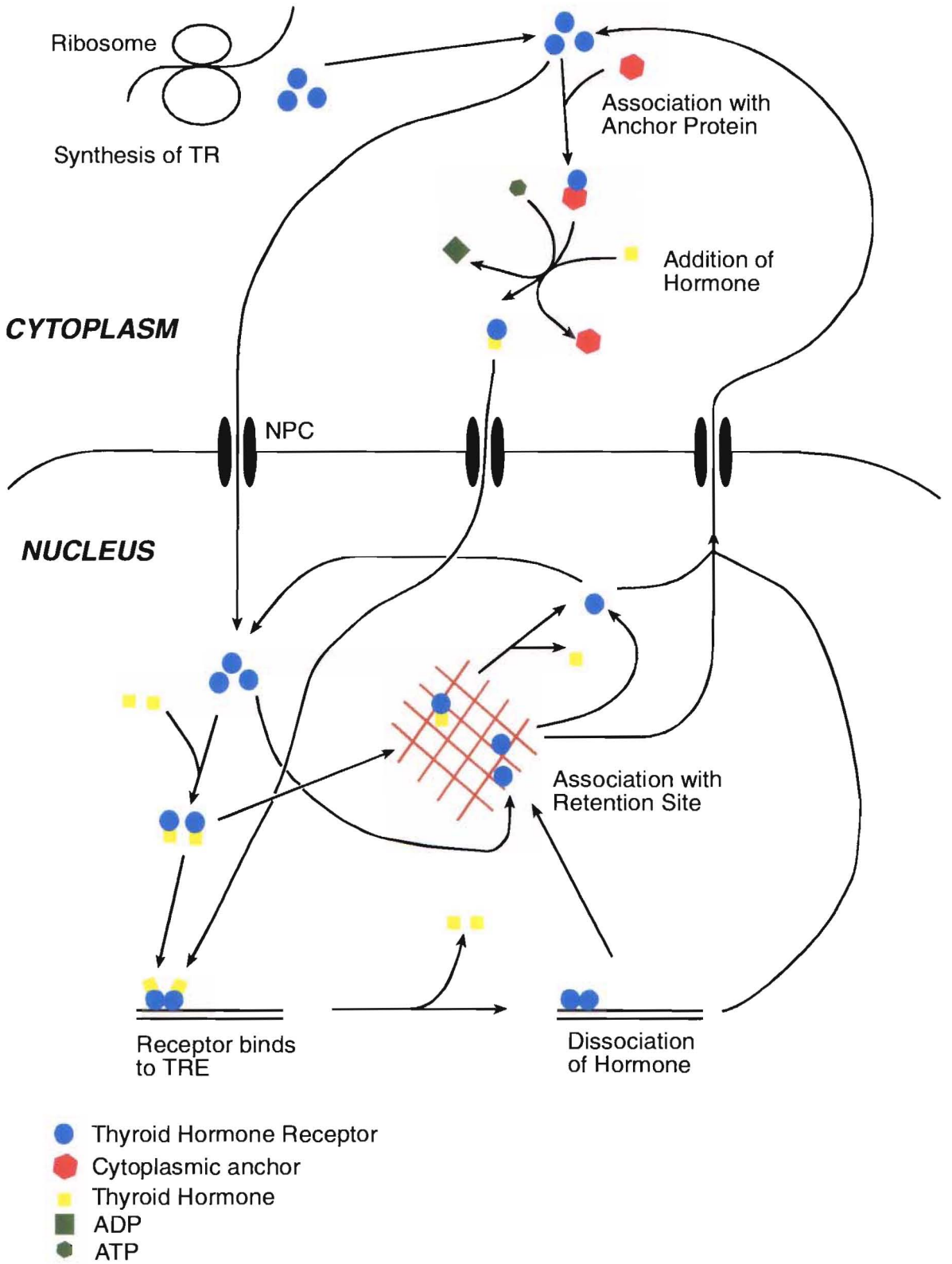


Figure 4.2. Model for interactions of TR with cellular components. ***See text for explanation (Section 4.2). Homodimers of TR are shown to bind TREs, however, heterodimers with RXR and RAR are also formed.***

4.3 Regulatory mechanisms of TR activity

A number of mechanisms have been proposed during the course of this study to account for regulation of TR activity. It seems likely that TR is regulated at a number of levels within the cell, in addition to regulation while bound to TREs. Perhaps the most 'global' mechanism is provided by the distribution of TR; that is, compartmentalisation of TR restricts access of these receptor molecules to activation sites, such as DNA and nuclear matrix, and factors involved in repression and activation at these sites. This compartmentalisation has been observed for several proteins as well as specifically for other members of the NHR superfamily. For example, several proteins have been observed to redistribute between the cytoplasmic and nuclear compartments in embryonic cells at particular developmental stages (reviewed in Nigg, 1990); and unliganded GR, AR, and MR appear restricted to the cytoplasm, due to complex formation with hsp90, until release by ligand binding, whereupon they localise to the cell nucleus (Yang and DeFranco, 1994).

Association with cytoplasmic anchoring factors is an aspect of this type of compartmental regulation. Regulation of nucleocytoplasmic transport by anchoring factors falls into two categories. Firstly, the cytoplasmic factor may act as a physical anchor. An example of this is the anchoring of sterol-regulatory element binding proteins and PKA holoenzyme by the ER and cytoplasmic membranous organelles (Wang *et al.,* 1994; Nigg, 1990; respectively). Secondly, the cytoplasmic anchor may mask the NLSs until modification of the anchor occurs, exposing the NLS. For example, IκB masks the NLS of NFκB/Rel, inhibiting import (Baeuerle and Henkel, 1994). IκB anchoring is inactivated by phosphorylation and proteolysis. As mentioned in the previous paragraph, several NHRs are physically anchored by the formation of complexes with hsp90. Although TR has not been shown to complex with hsp90 (Dalman *et al.,* 1991), it is possible that another anchoring factor is present in *Xenopus* oocytes, which would account for the presence of a large cytoplasmic population of TR in oocytes as opposed to somatic cells. Nuclear populations of TR, extracted from *Xenopus* oocytes by Dr L. A. Allison in pilot studies, are phosphorylated, unlike their cytoplasmic counterparts (personal communication). This observation could

indirectly support the presence of an anchoring protein, indicating that release of TR is dependent upon either the phosphorylation and modulation of TR itself or the phosphorylation of an NLS sequence. An alternative scenario is that TR shuttles through the nuclear and cytoplasmic compartments passively and is not retained or anchored in either compartment. This seems unlikely as TR export in oocytes has been shown to occur via a facilitated mechanism.

An extra level of regulation could be provided by nucleocytoplasmic transport. Regulation could be provided by the mechanism of transport or by the NPC itself. It has been shown that transport rates in proliferating cells are faster and the functional sizes of NPCs are significantly larger than in quiescent cells (Feldherr and Akin, 1994). Also, variability in the number of NPCs present in the nuclear membrane depends on the physiological state of the cell. Amphibian oocytes (such as *Xenopus* oocytes) have been estimated to have a density of over 60 pores per square micrometre, whilst most higher eucaryotes have a sparser density at 10 to 20 pores per square micrometre (reviewed in Nigg, 1990). Therefore, different mechanisms for TR transport might be in operation in different cell systems, dependent on the number of NPCs available. For example, in NIH 3T3 cells NPCs are sparser than in *Xenopus* oocytes and this might necessitate a facilitated mechanism to direct import.

The availability of nuclear binding or retention sites may also effect nuclear import. As discussed in Section 1.2.2, passive diffusion and facilitated diffusion occur down the chemical concentration gradient. Therefore, a protein imported by this mechanism may only continue to diffuse whilst there is a lower concentration of free protein in the nuclear binding. Protein that associates with nuclear binding sites, such as chromatin or nuclear matrix, is effectively removed from this concentration gradient. The significance of this is that once all of the binding sites in the nuclear compartment are taken, an equal concentration of free TR in both the nuclear and cytoplasmic compartments will be reached, providing a further level of regulation. Saturation of binding sites in the *Xenopus* oocytes was not observed (with 50 to 200 picograms introduced per oocyte) during the course of the investigations described in this thesis, although TR import occurred by a passive diffusion mechanism.

Further to the discussion of nuclear accumulation of TR, is the characterisation of nuclear binding sites in *Xenopus* oocytes and somatic cells. TR has been shown to be constitutively nuclear in somatic cells (Perlman *et al.,* 1982; Kumara-Siri *et al.,* 1986; Horowitz *et al.,* 1989; Puymirat *et al.,* 1989; Macchia *et al.,* 1992; Lee and Mahdavi, 1993), and in particular to be associated with the DNA in GH_1 cells (Perlman *et al.,* 1982). Between 30% and 50% of endogenous thyroid hormone-binding receptors in GC cells have also been shown to be associated with the nuclear matrix (Kumara-Siri *et al.,* 1986). However, the importance of this data is not clear as labelled T_3 molecules were tracked, and not receptor molecules themselves. Association of EGFP-tagged TRα with nuclear matrix components was also observed in NIH 3T3 cells (as presented in Section 2.3.7). The nuclear matrix may serve as a framework on which target DNA sequences are brought in contact with transcriptional regulatory proteins. These regulatory proteins include co-repressors (N-CoR and SMRT) and co-activators (including, steroid receptor co-activator-1, SRC-1, and transcription intermediary factor 2, TIF2), as well as regulators of these co-regulators (reviewed in Shibata *et al.*, 1997).

A significant shift in TR distribution (α and β isoforms) from the cytoplasmic to the nuclear compartments in *Xenopus* oocytes was induced by incubation in T_3. This shift was also observed for the DNA-binding mutant C122A, indicating that receptor does not just associate with DNA. These findings suggest that TR binds to at least two types of sites in cell nuclei (both in oocytes and somatic cells). TR binds to the TREs in DNA as well as to the nuclear matrix, although it was not determined whether TRE binding is necessary for nuclear matrix association. As discussed in Section 2.4.4, a two-site DNA model for NHR action has been proposed (reviewed in Nigg, 1990). The two types of sites for NHR nuclear accumulation would be a large number of low affinity sites or a lesser number of high affinity sites. All of these sites would not necessarily be functionally active binding sites, but rather may be retention sites or quasifunctional sites. Therefore, when applied to TR, DNA binding is observed and TR may also associate with a number of other sites with varying degrees of affinity. The increase in nuclear localisation of TR, induced by hormone, could reflect an increase the number of sites for potential binding, or may result in a rearrangement of TR distribution amongst these sites due to altered affinities. TR conformational changes have been shown to be induced by hormone binding (Toney *et al.*, 1993) and it has been reported

that T_3 induced an enhanced rate of translocation of EGFP-tagged TRβ to the nuclei of CV1 cells (Zhu *et al.*, 1998).

4.4 Prospects and conclusions

The data contained in this thesis suggest that TR localisation occurs by a process regulated at several levels. Regulation of TR function does not only occur at the transcriptional regulation level, but rather involves transport mechanisms that seem to be regulated by hormone binding and possibly by phosphorylation. The findings in *Xenopus* oocytes are novel and do not reflect the previously described constitutively nuclear state of TR in somatic cells.

Thyroid hormone is involved in the growth and developmental processes in virtually every type of vertebrate tissue (reviewed in Chatterjee and Tata, 1992), and it is TR that regulates the transcription of the genes that mediate the effects of T_3 on differentiation, development, and metabolism (Ribeiro *et al.*, 1995). Therefore, mutations in TR could underlie T_3-linked diseases in humans (Nagaya and Jameson, 1993). Aberrant expression of TR has been implicated in a variety of endocrine and neoplastic diseases, such as small cell lung carcinoma, generalised thyroid hormone resistance, and colon carcinoma in humans, as well as hepatocellular carcinoma in mice (Dobrovic *et al.*, 1988; Markowitz *et al.*, 1989; Damm, 1993; Barlow *et al.*, 1994; Yen *et al.*, 1994; Beug *et al.*, 1996; Fraichard *et al.*, 1997; Lin *et al.*, 1997). Characterisation of TR nuclear localisation aids in defining the regulatory mechanisms in operation for this NHR. Knowledge of how proteins function, and are regulated, in the normal cell is important in assisting us to elucidate the changes that occur upon disruption of the normal environment. Only by understanding these changes can we better formulate treatments against the central defects of a transformed cell.

The investigations presented in this thesis suggest a variety of directions for future studies of TR and its variants. Whilst the focus of this thesis has fallen on TR, transport of v-ErbA is an obvious direction for future research. The v-*erbA* oncogene is unique, but similar derivatives of TR might be involved in human cancers. Therefore, the viral oncogene

provides us with a tool for investigating disruption of normal cell function. The v-*erbA* gene is fused to the viral gene, *gag*, which accounts for the greatly increased receptor size. Separation of the two component sequences would enable assignment of transport and subcellular localisation characteristics to each of the two gene products. Interestingly, v-ErbA was localised to the nuclear membrane and cytoplasmic fibrils of NPCs in *Xenopus* oocytes suggesting a role in modulating nucleocytoplasmic transport (Nagl *et al.*, 1997).

The scope for further investigation of TR is broad and could encompass the interactions of TR with cytoplasmic anchors, elucidation of NLS and NES sequences and function, characterisation of alternative nuclear binding sites and their relative affinities, and the export and nucleocytoplasmic shuttling of receptor molecules. Confirmation of nucleocytoplasmic shuttling in mammalian cells would aid in the interpretation of results contained in this thesis, and therefore pilot studies in heterokaryon cells are being carried out in Dr L. A. Allison's laboratory (College of William and Mary).

The field of investigation of NHR distribution and nucleocytoplasmic trafficking is very much influenced by the available techniques. One has only to examine the findings made in this field in the last decade to appreciate its rapid advancement. Perhaps most importantly, these advances emphasise the dynamic nature of biological systems and their ability to respond to a wide range of stimuli in a regulated manner.

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Appendix I

Protein sequence comparison of TR and variants

hTRβ[1] .....

rTRα[2] .....

gag-v-erbA[3] *reeqvtseqakfwlglgggrvsppgpeciekpaterridkgeemg*

hTRβ .....

rTRα .....

gag-v-erbA *ettvqrdakmapekmatpktvgtscyqcgtatgcncvtasappppyvgsglypslagage*

hTRβ mtengltawdkpkhcpdrehdwklvgmseaclhrkshserrstlkneqssphliqttwts

rTRα meqkpskvecg

gag-v-erbA *qggqggdtprgaeqpraepghagqapgpaltdwarireelastgppvvampvviktegpaw*

hTRβ sifhldhddvndqsvssaqtfqteekkckgyipsyldkdelcvvcgdkatgyhyrcitce

rTRα sdpeensarspdgkrkrkngqcplkssmsgyipsyldkdeqcvvcgdkatgyhyrcitce

gag-v-erbA *tplepedtrwl*dgkhkrkssqclvkssmsgyipscldkdeqcvvcgdkatgyhyrcitce

hTRβ* gckgffrrtiqknlhpsysckyegkcvidkvtrnqcqecrfkkciyvgmatdlvlddskr

rTRα gckgffrrtiqknlhptysckydsccvidkitrnqcqlcrfkkciavgmamdlvlddskr

gag-v-erbA gcksffrrtiqknlhptysctydgccvidkitrnqcqlcrfkkcisvgmamdlvlddskr

hTRβ .....

rTRα .....

gag-v-erbA vakrklieenrerrrkeemikslqhrpspsaeewelihvvteahrstnaqgshwkqrrkf

hTRβ lpedigqapivnapeggkvdleafshftkiitpaitrvvdfakklpmfcelpcedqiill

rTRα lpddigqspivsmpdgdkvdleafseftkiitpaitrvvdfakklpmfselpcedqiill

gag-v-erbA lledigqspmasmldgdkvdleafseftkiitpaitrvvdfaknlpmfselpcedqiill

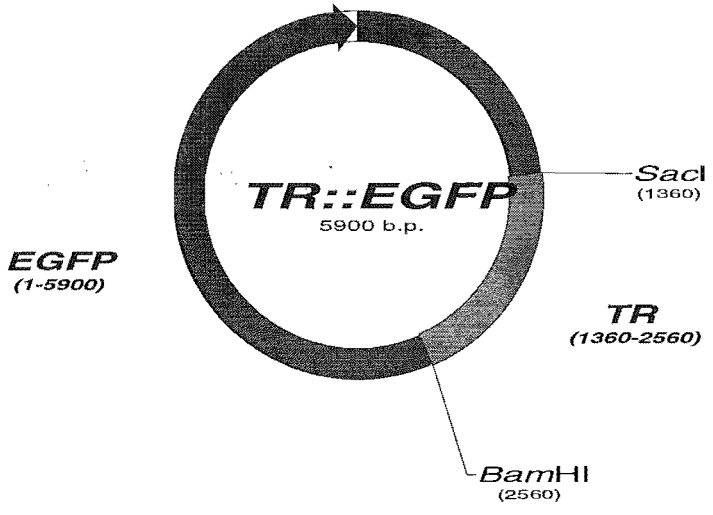
```
hTRβ          kgccmeimslraavrydpesetltlngemavirgqlkngglgvvsdaifdlgmslssfnl
rTRα          kgccmeimslraavrydpesdtltlsgemtvkrkqlkngglgvvsdaifelgkslsafnl
gag-v-erbA    kgccmeimslraavrydpesetltlsgemavkreqlkngglgvvsdaifdlgkslsafnl

hTRβ          ddtevallqavllmssdrpglacveriekyqdsfllafehyinyrkhhvthfwpkllmkv
rTRα          ddtevallqavllmstdrsgllcvdkieksqeayllafehyvnhrkhniphfwpkllmkv
gag-v-erbA    ddtevallqavllmssdrtglicvdkiekcqesyllafehyinyrkhniphfwskllmkv

hTRβ          tdlrmigachasrflhmkvecptellpplflevfed
rTRα          tdlrmigachasrflhmkvecptelfpplflevfedqev
gag-v-erbA    adlrmigayhasrflhmkvecptelspqev
```

1 hTRβ accession number 72119
2 rTRα accession number 112393
3 gag-v-erbA accession number 871521

* denotes the line in which a cysteine (c) to alanine transition occurs in C122A

Appendix II

PEGFP::TRα construct

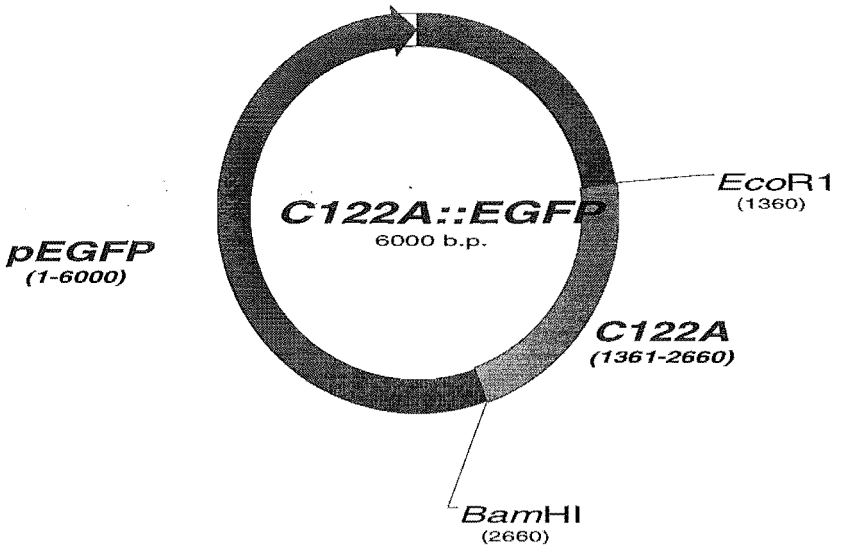


PCR primers:

Left	CC	GAGCTCG	A	ATGGAACAGAAGCC
(function)	link	SacI	link	insert
Right	GGT	GGATCC	TTA	GACTTCCTGATCC
(function)	vector	BamHI	stop	insert

Appendix III

pEGFP::C122A construct



PCR primers:

Left	CCC GAATTC G ATGACAGAAAATGGCC
(function)	vector EcoRI link insert
Right	GGT GGATCC CTA ATCCTCGAACACTTCC
(function)	vector BamHI stop insert

Appendix IV

This reprint from the *Journal of Cellular Biochemistry* contains results obtained during the initial oocyte investigations described in this thesis. My contributions to the paper included the microinjection and analysis of the oocytes in the experiment detailed in Fig. 1. (C) and the microinjection of oocytes for the experiment detailed in Fig. 2. (B). I also assisted Dr S. Nagl in the processing and analysis of these oocytes.

Journal of Cellular Biochemistry 67:184–200 (1997)

v-*erbA* Oncogene Initiates Ultrastructural Changes Characteristic of Early and Intermediate Events of Meiotic Maturation in *Xenopus* Oocytes

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Abstract The growth-promoting properties of the retroviral v-*erbA* oncogene, a highly mutated version of the chicken thyroid hormone receptor (TR) α, have so far exclusively been linked to dominant repression of the antimitogenic roles of TR and retinoic acid receptors. Here we show that when expressed in *Xenopus* oocytes v-ErbA induced ultrastructural changes characteristic of early and intermediate events of meiotic maturation by activating gene transcription. v-ErbA–induced maturation events occurred without activation of the cAMP/maturation-promoting factor signal pathway and were arrested prior to meiotic spindle formation. The effects of v-ErbA were not mimicked by a dominant negative in vitro–generated mutant of human TR, suggesting that v-ErbA can contribute to cell cycle reentry by interference with regulatory pathways distinct from those involving TR. Interestingly, a portion of v-ErbA expressed in oocytes was present at the cytoplasmic fibrils of the nuclear pore complexes, suggesting that in addition to its intranuclear function v-ErbA may modulate nucleocytoplasmic transport. J. Cell. Biochem. 67:184–200, 1997.

Key words: maturation-promoting factor; meiosis; nuclear pore complex; nucleocytoplasmic transport; thyroid hormone receptor

The cooperative action of v-*erbA* and its retroviral partner v-*erbB*, both carried by the avian erythroblastosis virus, induces acute erythroblastosis and fibrosarcomas in young chicks. The v-*erbA* product, a highly mutated version of the chicken thyroid hormone receptor (TR) α, dominantly represses the actions of normal TR and retinoic acid receptors (RARs) [reviewed in Privalsky, 1992]. In addition, two mutations in the DNA binding domain, together with mutations in an N-terminal region, alter the range of DNA target sequences the v-*erbA* protein can bind to relative to TR [Chen et al., 1993; Smit-McBride and Privalsky, 1993; Subauste and Koenig, 1995]. While chicken embryo fibroblasts expressing only v-*erbA* do not display a fully transformed phenotype, they exhibit a greatly enhanced growth potential and a decreased requirement for growth factors [Gandrillon et al., 1987].

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The growth-promoting properties of v-ErbA have so far been linked to dominant repression of the antimitogenic roles of TR and RAR. This mode of action is indicated by the fact that v-*erbA* is able to stimulate proliferation by overcoming growth inhibition by retinoic acid [Sharif and Privalsky, 1991]. In this context, v-*erbA* function is strongly correlated with its action as a dominant negative oncogene in abolishing AP-1 repression by RAR and TR [Desbois et al., 1991a,b; Zhang et al., 1991]. Furthermore, in the presence of v-*erbA*, erythrocytic progenitor cells are insensitive to the induction of apoptosis and self-renewal inhibition by retinoic acid or thyroid hormone [Gandrillon et al., 1994]. The question of whether the growth-promoting properties of v-ErbA can be exclusively attributed to dominant repression of TR and RAR or whether they also involve the activation of specific v-ErbA–responsive genes remains unanswered to date. Thus, we sought to determine whether it was possible to identify a direct role of v-ErbA in cell cycle induction at the level of gene transcription by employing meiotic maturation assays.

Meiotic maturation assays carried out in fully grown (stage VI) [Dumont, 1972] oocytes from

Xenopus laevis, the African clawed frog, have been used extensively for studies of oncogene involvement in the kinase signal cascades of the cell cycle [reviewed in Smith, 1989]. Fully grown oocytes are arrested at the G_2/M border and can be induced to enter M-phase by progesterone, insulin, and a range of introduced oncogenic protein kinases. Within minutes of progesterone exposure, a decrease in levels of cAMP occurs, mediated by inhibition of adenylate cyclase, which in turn downregulates protein kinase A and protein kinase C activities [Smith, 1989]. The induction of *Xenopus* oocyte maturation by progesterone does not require gene transcription but is absolutely dependent on polyadenylation and translation of stored mRNA coding for the serine-threonine kinase Mos [Baltus et al., 1973; Sagata et al., 1988; Yew et al., 1992; Sheets et al., 1995]. A network of phosphorylation cascades [Kosako et al., 1994a,b; reviewed in Mordret, 1993] culminate in the activation of maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK). MPF contains the *Xenopus* homolog of the universal M-phase inducer $p34^{cdc2}$, a serine-threonine kinase with histone H1 phosphorylating activity, which is complexed with B-type cyclins [Maller, 1990]. MPF and MAPK trigger the events of maturation, including nuclear breakdown, chromosome condensation, and meiotic spindle formation [Smith, 1989; Bement and Capco, 1990].

In fully grown (stage VI) oocytes, the chromosomal loops associated with high transcriptional activity during earlier stages of oogenesis have retracted considerably, and the chromatin is quite condensed [Hausen and Riebesell, 1991]. Thus, although microinjected gene templates are transcribed efficiently in stage VI oocytes [Nagl et al., 1995], it was an open question whether *Xenopus* homologs of potential target genes would be accessible to v-ErbA and the general transcription machinery in stage VI oocytes.

In the present study, we show that v-ErbA initiates ultrastructural changes characteristic of early and intermediate meiotic events, but meiosis was arrested prior to spindle formation. The initiation of this subset of maturation events by v-ErbA required de novo gene expression and did not involve activation of the cAMP/MPF signal pathway. Events induced by v-ErbA were not mimicked by a dominant negative in vitro–generated mutant of TR, suggesting that the ultrastructural changes were not mediated by dominant repression of endogenous TR. Our study suggests that v-ErbA, acting as a transcriptional activator, can contribute to cell cycle reentry by interference with regulatory pathways distinct from those involving TR.

MATERIALS AND METHODS

Plasmids

RS-v-*erbA* was a gift from R. Evans (Salk Institute for Biological Studies, La Jolla, CA). The expression vector contains the *gag*-v- *erbA* oncogene cDNA under the transcriptional control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) [Damm et al., 1989; Thompson and Evans, 1989]. The RSh-TRβ C122>A expression plasmid was a gift of P. Romaniuk (University of Victoria, Victoria, BC, Canada). It contains an in vitro–generated mutant human TRβ gene (alanine instead of cysteine at position 122) under control of the RSV LTR [Nelson et al., 1993]. pRSV-*lacZ* was obtained from M. Harkey (University of Washington, Seattle, WA) and contains the *E. coli* β-galactosidase gene under control of the RSV LTR [Gorman et al., 1983]. The pKCR2-cea plasmid was a gift of B. Vennström (Karolinska Institute, Stockholm, Sweden) and contains the chicken *c-erbA* cDNA expressed from the SV-40 promoter [Sap et al., 1989]. The pGEM-4Z vector was a gift of M. Privalsky (University of California, Davis, CA), and contains the *gag*-v-*erbA* cDNA under control of the SP6 RNA polymerase promoter.

Oocyte Microinjections

A lobe of ovary was surgically removed from an adult female *Xenopus laevis* and processed as described [Allison et al., 1991, 1993]. Microinjections were performed according to published methods with modifications [Nagl et al., 1995]. Defolliculated stage VI oocytes [Dumont, 1972] were microinjected with 5 ng expression vector in a fixed volume of 20 nl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) into the nucleus by the "blind" injection method with the needle inserted in the center of the animal pole. Alternatively, v-*erbA* protein was synthesized from the pGEM-4Z template by in vitro coupled transcription/translation in rabbit reticulocyte lysate (Promega, Madison, WI), according to the manufacturer's specifications, using SP6 RNA polymerase. Fifty nanoliters v-*erbA* solution (approximately 100 pg v-ErbA in rabbit reticulo-

cyte lysate) was microinjected into the oocyte cytoplasm.

Incubation Treatments and Assessment of Germinal Vesicle Migration and Germinal Vesicle Breakdown

Oocytes were incubated in O-R2 medium [Allison et al., 1991] only or in O-R2 containing (1) 10 μg/ml progesterone (Sigma Chemical Co., St Louis, MO), (2) 20 μM forskolin (Sigma) and 2 mM isobutylmethylxanthine (IBMX) (Sigma); (3) 30 μg/ml actinomycin D (actinomycin C_1) (Boehringer Mannheim NZ Ltd., Auckland, New Zealand), or (4) 200 μg/ml cycloheximide (BDH Chemicals NZ Ltd., Palmerston North, New Zealand). Twenty-four hours after nuclear microinjection with 5 ng RS-v-*erbA* or pRSV-*lacZ* or cytoplasmic injection of 50 nl of in vitro–synthesized v-*erbA* protein, oocytes were scored for germinal vesicle migration (GVM) under a stereomicroscope, as indicated by pigment rearrangement at the apical pole that produces a whitish circular spot delineated by a dark ring of pigment [Smith, 1989; Bement and Capco, 1990; Brachet et al., 1970]. Oocytes were assessed for germinal vesicle breakdown (GVBD) by manual dissection after fixation in ice-cold 1% trichloroacetic acid (TCA) for 5 min. Oocytes were scored positive for GVBD when the nucleus could not be dissected intact due to nuclear instability.

Differential Interference Contrast Microscopy

Oocytes incubated in progesterone or microinjected with 5 ng RS-v-*erbA*, RSh-TRβ C122 > A, or pRSV-*lacZ* were fixed and embedded essentially as described by Hausen and Riebesell [1991]. In brief, oocytes were fixed in Romeis fixative (25 ml saturated mercuric chloride, 20 ml 5% TCA, 15 ml 37% formaldehyde) for 3 h. Subsequently, oocytes were dehydrated in an ethanol series of 50% (15 min), 70% (30 min), 80% (15 min), 90% (15 min), 95% (15 min), and 100% (3 × 10 min), infiltrated in 50% glycol methacrylate infiltration solution (Polaron, Auckland, New Zealand) in ethanol for 1 h, and then left in 100% infiltration solution overnight. Oocytes were embedded in glycol methacrylate (Polaron) in gelatine capsules under nitrogen at 37°C for 5 h. Five micron sections were obtained with Ralph glass knives. In order to remove mercury precipitates, we treated the dry sections with an alcoholic iodine potassium iodide solution (2% iodine, 3% potassium iodide in 90% ethanol) for 2 min, washed them in 0.25% sodium thiosulfate for 15 min, and thoroughly rinsed them in distilled water. The sections were stained utilizing an azofuchsin/aniline blue/orange G triple-staining method [Hausen and Riebesell, 1991]. Three independent batches of oocytes were used, and three to four oocytes per treatment per batch were analyzed using differential interference contrast microscopy. Oocyte sections were photographed using Agfa optima color film with a blue filter.

Electron Microscopy for Ultrastructural Analysis

In ultrastructural studies of *Xenopus* oocytes, long fixation times and special fixation procedures are necessary. Unless indicated otherwise, all procedures were carried out at 4°C. Oocytes were fixed in 2.5% glutaraldehyde and 0.05% low molecular weight tannic acid in O-R2 overnight, washed in O-R2 three times for 30 min, and subsequently transferred to 2% OsO_4 (in ddH_2O) for 3 h with one change. Oocytes were then washed in O-R2 for 30 min and dehydrated in a graded ethanol series of 30% (30 min), 50% (30 min), 70% (overnight), 80% (20 min), 90% (20 min), 95% (15 min), and 100% (2 × 15 min). After the 70% ethanol step, all further procedures were carried out at room temperature. Oocytes were incubated in 100% acetone twice for 20 min and then gradually embedded in Spurr's resin in acetone: 30% resin (1 h), 50% resin (3 h), 70% resin (overnight), 90% resin (8 h), and 100% resin (overnight). Oocytes were transferred into 100% resin in molds and cured at 70°C for 24 h. Ultrathin sections (50 nm) were stained with 5% uranyl acetate in 50% ethanol for 20 min, washed in ddH_2O, stained with lead citrate for 20 min, and put through a final washing step in ddH_2O. Three independent batches of oocytes were used, and three to four oocytes per treatment per batch were analyzed.

Histone H1 Kinase Assay

Adult female *Xenopus laevis* were injected with 0.5 ml gonadotropin (from pregnant mares' serum (Sigma); 200 U/ml in sterile ddH_2O) subcutaneously into the dorsal lymph sac to improve the synchrony of response to progesterone in oocytes. After 4 days, a lobe of ovary was surgically removed from the animal and processed as described above. Defolliculated stage VI oocytes were microinjected into the nucleus with 5 ng RS-v-*erbA* or pRSV-*lacZ* or incubated in 10 μg/ml progesterone. At specified times, oocyte extracts were prepared by homogenizing

groups of ten oocytes in 20 µl extraction buffer (20 mM Tris, pH 7.5, 80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 25 µg/ml aprotinin, 25 µg/ml leupeptin, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM Pefabloc (Boehringer Mannheim), and 1 mM DTT). The homogenate was centrifuged at 10,000*g* for 10 min at 4°C to pellet yolk and pigment. Ten microliters of the supernatant were mixed and incubated for 20 min at 25°C with 10 µl kinase buffer (30 mM Tris, pH 7.5, 30 mM $MgCl_2$, 1 mCi/ml γ[^{32}P] ATP (Amersham Life Sciences, Auckland, New Zealand), 3 mg/ml histone H1 [Sigma]) [Kosako et al., 1994b]. The reaction was stopped by the addition of an equal volume of 2× SDS sample buffer (4% SDS, 20% glycerol, 120 mM Tris, pH 6.8, 0.01% bromophenol blue) and boiling for 5 min. Samples were resolved by 12% SDS-PAGE followed by autoradiography.

Immunoprecipitation of Oocyte Fractions With an Anti-v-erbA Antibody

Oocytes were microinjected with 5 ng RS-v-*erbA* or pKCR2-cea into the nucleus and incubated in a sterile microtitre plate (five oocytes/well) in 30 µl of O-R2 with 1 mCi/ml L-[^{35}S] methionine (1,000 Ci/mmol) (Amersham) at 18°C for 24 h. Nuclear fractions of 10 or 20 pooled v-*erbA*–injected oocytes were prepared manually, after fixation in 1% ice-cold TCA for 5 min [Allison et al., 1993]. Net-2 (50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Nonidet P-40) samples were homogenized in 0.5 ml NET-2 [Allison et al., 1993] containing 0.1 mM PMSF. For preparation of nuclear membrane fractions, nuclei were manually isolated in nuclear isolation medium (NIM) (83 mM KCl, 17 mM NaCl, 10 mM Tris, pH 7.2, 5 mM Pefabloc) [Krohne and Franke, 1983] in a small siliconized petri dish using watchmaker's forceps. Nuclei were freed from adhering yolk by aspirating them into a pipette with an inner bore diameter of 0.7–0.8 mm and then washed once in fresh NIM and transferred into nuclear envelope isolation medium (NEM) (83 mM KCl, 17 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris, pH 7.2, 5 mM Pefabloc). Within 30–60 s, the nuclear content forms an opaque and compact aggregate from which the nuclear membrane (envelope) detaches. The nuclear membranes were isolated by aspirating nuclei into a pipette with an inner bore diameter of 0.3–0.4 mm, cleaned in fresh NEM by repeated aspiration into a small pipette, and transferred into a microfuge tube. Ten or 20 nuclear envelopes were pooled per sample and collected by centrifugation for 4 min at 8,000*g* and resuspended by gentle aspiration in 0.5 ml NET-2 containing 5 mM Pefabloc.

For total soluble protein electrophoretic separations, 20 µl 2× SDS sample buffer and 1 mM dithiothreitol (DTT) were added to 20 µl cleared homogenate from nuclear and nuclear membrane fractions, which were then resolved by 12% SDS–polyacrylamide gel electrophoresis and stained with Bio-Rad Silver Stain (Bio-Rad Laboratories Pty Ltd., Auckland, New Zealand).

For immunoprecipitation assays, 30 µl of monoclonal antibody LAO38 against v-*erbA* residues 58–75 (Quality Biotech, Camden, NJ) were bound to 20 µl preswollen protein G–Sepharose beads (Gamma Bind Plus Sepharose, Pharmacia LKB Biotechnology, Auckland, New Zealand) in 0.45 ml NET-2 for 2 h at room temperature with end-over-end rotation. After incubation, the resin was pelleted for 5 s in a microfuge and resuspended in 1 ml of NET-2, and the wash was repeated three times. Pooled nuclei or nuclear membrane samples in NET-2 were added to each washed protein G–Sepharose-antibody pellet and incubated at 4°C for 1 h with end-over-end rotation. After incubation, the resin with bound antigen was pelleted and washed four times with NET-2. Twenty microliters of 2× SDS sample buffer was added to each pellet, the samples were heat-denatured to release the antigen, and the resin was pelleted for 5 s in a microfuge. The supernatant (with 1 mM DTT added) was resolved by 12% SDS-PAGE followed by fluorography.

Immunolabeling of Isolated Nuclear Membranes

Oocytes were microinjected with 5 ng RS-v-*erbA* into the nucleus and incubated in O-R2 for 12–14 h. Nuclear membranes of 20 oocytes per sample were manually isolated as described above and fixed in 2% formaldehyde, freshly made from paraformaldehyde in phosphate buffered saline (PBS) (137 mM NaCl, 1.3 mM Na_2HPO_4, 2.5 mM NH_2PO_4, pH 7.2), for 10 min at 4°C. Immunolabeling with colloidal gold, employing a biotin-streptavidin bridging technique, was carried out following published procedures [Cordes et al., 1993] with modifications and according to the manufacturer's specifications (Amersham). Membranes were collected by centrifugation at 8,000*g* for 4 min and washed three times in PBS, pH 7.2, containing

50 mM NH_4Cl. Membrane samples were then resuspended in 24 µl PBS, pH 7.2, with 6 µl anti-v-*erbA* antibody LA038, and incubated for 2 h at room temp. Subsequently, membranes were centrifuged at 8,000*g* for 4 min, washed three times in PBS, resuspended in 99 µl PBS, pH 7.2, containing 1 µl biotinylated goat anti-mouse Ig antibody (Amersham), and then incubated for 2 h at room temperature. The samples were then washed with PBS, pH 7.2, as described above. Next, membranes were incubated in 38 µl PBS, pH 7.2, with 2 µl AuroProbe EM streptavidin 10 nm (Amersham) for 2 h at room temperature and washed in PBS as before. Membranes were fixed in 2.5% glutaraldehyde in PBS, pH 7.2, for 30 min at 4°C, washed three times in PBS, and treated with 2% aqueous OsO_4 for 30 min at 4°C, followed by several

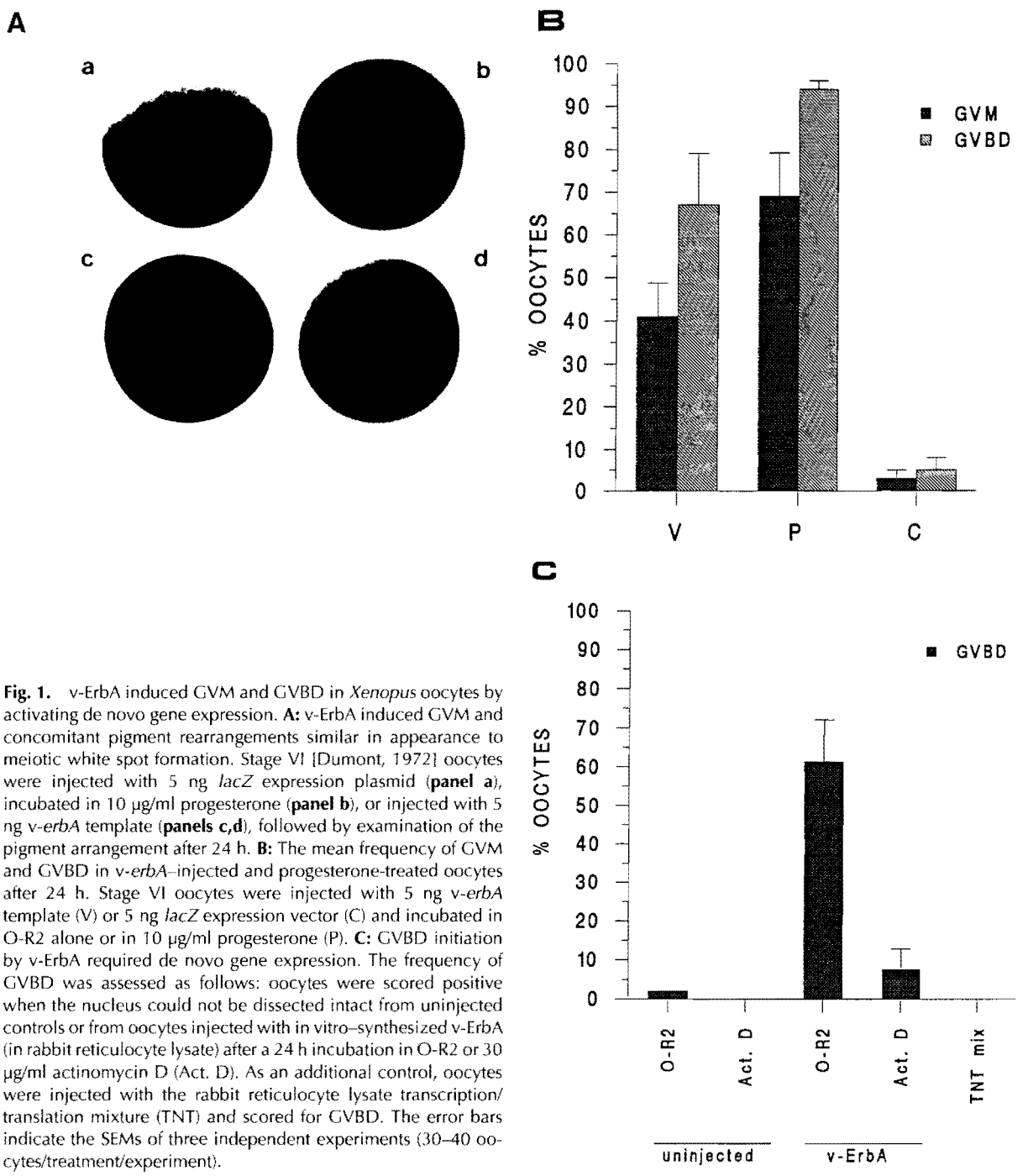

Fig. 1. v-ErbA induced GVM and GVBD in *Xenopus* oocytes by activating de novo gene expression. **A:** v-ErbA induced GVM and concomitant pigment rearrangements similar in appearance to meiotic white spot formation. Stage VI [Dumont, 1972] oocytes were injected with 5 ng *lacZ* expression plasmid (**panel a**), incubated in 10 µg/ml progesterone (**panel b**), or injected with 5 ng v-*erbA* template (**panels c,d**), followed by examination of the pigment arrangement after 24 h. **B:** The mean frequency of GVM and GVBD in v-*erbA*–injected and progesterone-treated oocytes after 24 h. Stage VI oocytes were injected with 5 ng v-*erbA* template (V) or 5 ng *lacZ* expression vector (C) and incubated in O-R2 alone or in 10 µg/ml progesterone (P). **C:** GVBD initiation by v-ErbA required de novo gene expression. The frequency of GVBD was assessed as follows: oocytes were scored positive when the nucleus could not be dissected intact from uninjected controls or from oocytes injected with in vitro–synthesized v-ErbA (in rabbit reticulocyte lysate) after a 24 h incubation in O-R2 or 30 µg/ml actinomycin D (Act. D). As an additional control, oocytes were injected with the rabbit reticulocyte lysate transcription/translation mixture (TNT) and scored for GVBD. The error bars indicate the SEMs of three independent experiments (30–40 oocytes/treatment/experiment).

washes in ddH_2O. Membrane samples were dehydrated in a graded ethanol series, incubated in 100% acetone twice for 20 min, and embedded in Spurr's resin in acetone: 50% resin (1 h), 90% resin (overnight), and 100% resin (1 h). Membranes were then immersed in fresh resin and cured at 70°C for 14 h. Ultrathin sections (50 nm) were stained with 2% uranyl acetate in 50% ethanol for 10 min, washed in ddH_2O, stained with lead citrate for 5 min, and washed in ddH_2O.

RESULTS

v-ErbA Induced GVM and GVBD in *Xenopus* Oocytes by Activating De Novo Gene Expression

During meiosis, pigment granules in the animal hemisphere cortex of *Xenopus* oocytes are rearranged, producing a whitish circular spot [reviewed in Smith, 1989]. Pigment displacement commences during early maturation and is thought to be initiated by structural changes in the oocyte cortex immediately upon meiotic induction [Bement and Capco, 1990]. The progressive migration of the nucleus (germinal vesicle migration), from near the center of the oocyte toward the animal pole, contributes to the rearrangement of pigment granules. Upon germinal vesicle breakdown and completion of maturation, a clearly delineated white spot represents the area where the meiotic spindle has formed [reviewed in Smith, 1989; Bement and Capco, 1990; Brachet et al., 1970].

We first tested the ability of v-ErbA to initiate maturation in *Xenopus* oocytes by comparing the rate of nuclear migration and breakdown in progesterone-treated and v-*erbA*–injected oo-

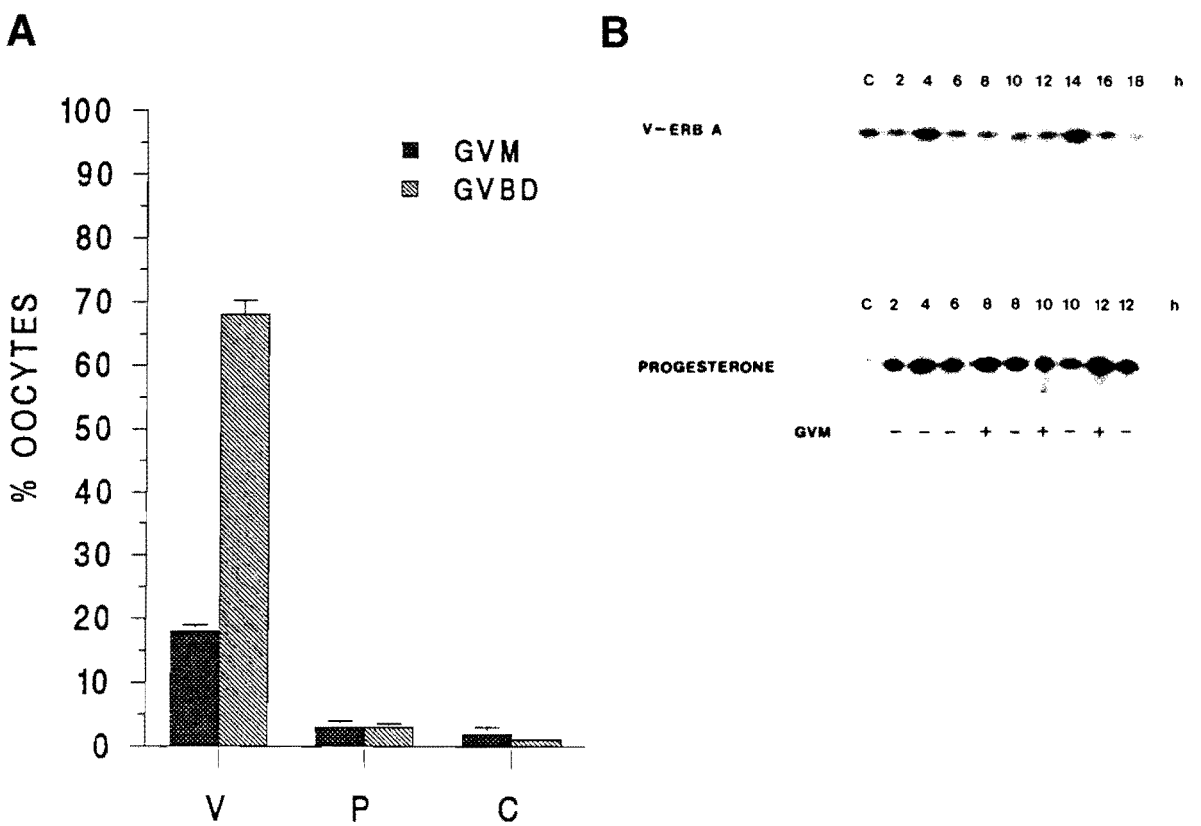

Fig. 2. The effects of expression of v-*erbA* in oocytes occurred independently of the cAMP/MPF pathway. **A:** v-*erbA*–injected (V), *lacZ*-injected (C), or progesterone-treated (P) oocytes were incubated in 20 μM forskolin/2 mM IBMX. After 24 h, the percentage of oocytes displaying GVM was assessed by inspection of whole oocytes, and the frequency of GVBD induction was scored by manual dissection of nuclei in 1% TCA. Three independent experiments were performed (30–40 oocytes per treatment per experiment). The error bars indicate the SEMs. **B:** v-ErbA did not induce MPF histone H1 kinase activity. After microinjection of 5 ng v-*erbA* or pRSV-*lacZ* template as a control (C) (**upper panel**) or incubation in progesterone or O-R2 as a control (C) (**lower panel**), oocytes were collected at the indicated times, and MPF kinase activity was assayed using histone H1 as a substrate and $\gamma[^{32}P]$ ATP. Samples were resolved by 12% SDS-polyacrylamide gel electrophoresis followed by autoradiography. MPF histone H1 kinase activity was assayed in oocytes with (+) or without (−) a distinct white spot (GVM). A representative example of three independent experiments, using oocytes from different female *Xenopus*, is shown.

cytes obtained from the same females. We have shown previously that v-ErbA, synthesized from a microinjected gene template in *Xenopus* oocytes, is functional and acts as a dominant repressor of TR [Nagl et al., 1995]. Microinjection of v-*erbA* expression plasmid into the nucleus of stage VI oocytes resulted in GVM and concomitant pigment displacement which, in most cases, was highly similar in appearance to GVM induced by progesterone after 24 h (Fig. 1A; cf. panels b,c). Occasionally, pigment aggregation in the center of the whitish circular spot in v-*erbA*–injected oocytes was seen (Fig. 1A, panel d). GVM occurred in 41% of v-*erbA*–injected oocytes, while the mean frequency of GVM in progesterone-stimulated oocytes was 69% (Fig. 1B). Next, oocytes were assessed for GVBD and were scored positive when the nucleus could not be dissected intact due to nuclear instability 24 h after microinjection of v-*erbA* template or due to nuclear dissolution after a 24 h incubation in progesterone. According to this criterion, GVBD occurred at a mean frequency of 67% in v-*erbA*–injected oocytes compared to 94% in progesterone-treated oocytes (Fig. 1B). It has been noted previously that the pigment displacement associated with GVM is not always apparent during maturation [Smith, 1989; Bement and Capco, 1990]. In the present study, GVBD could be observed in the absence of GVM both in progesterone-treated and v-*erbA*–injected oocytes.

It should be noted that progression through meiosis, induced by progesterone, occurred at a decelerated pace in all oocytes employed in this study [for comparison see Smith, 1989; Bement and Capco, 1990]. We attribute this fact to seasonal and dietary factors in *Xenopus* donor animals. Since this time course was consistent throughout the study, this did not interfere with the temporal comparison of progesterone- and v-*erbA*–induced events. Progesterone-treated oocytes, scored positive for GVBD, still retained remaining fragments of the germinal vesicle after 24 h. In contrast, when v-*erbA*–injected oocytes were incubated in progesterone, the complete disappearance of the nucleus was observed in all oocytes examined upon manual dissection after 24 h (data not shown). This shortened time course for the complete breakdown of the nucleus may suggest synergistic action of v-ErbA and progesterone in meiotic maturation.

To test whether the partial release of G_2 arrest by v-ErbA required de novo gene expression, we scored the frequency of GVBD in oocytes that had been injected with in vitro–synthesized v-*erbA* protein into the cytoplasm or treated with progesterone and incubated in actinomycin D. Consistent with the reported lack of requirement for gene transcription during progesterone-mediated meiotic induction [Baltus et al., 1973; Sagata et al., 1988; Yew et al., 1992; Sheets et al., 1995], actinomycin D did not affect the frequency of GVBD in progesterone-treated oocytes (data not shown). Since v-*ErbA* was not purified from the translation reaction mixture, it was possible that the rabbit reticulocyte lysate could supply factors that would effect GVBD. Thus, oocytes were injected with lysate mixture as a control. As shown in Figure 1C, the lysate alone did not induce GVBD. Twenty-four hours after microinjection, the mean frequency of GVBD in oocytes injected with v-ErbA and incubated in the absence of inhibitor was 61% in this series of experiments (Fig. 1C). In contrast, incubation of v-ErbA–injected oocytes in actinomycin D reduced the frequency of GVBD to 7.5% (Fig. 1C), showing that the nuclear migration and nuclear instability induced by v-ErbA were dependent on de novo gene expression. A requirement for translation of the newly transcribed message(s) was shown by reduction of GVBD frequency to the same level (i.e., 7.5%), by cycloheximide (data not shown).

Induction of GVM and GVBD by v-ErbA Does Not Involve Activation of the cAMP/MPF Pathway

Treatment of oocytes with forskolin, an adenylate cyclase activator, or IBMX, an inhibitor of cAMP breakdown, blocks meiotic induction by progesterone [reviewed in Smith, 1989; Bement and Capco, 1990]. Forskolin and IBMX did not significantly affect GVBD in v-*erbA*–injected oocytes and decreased GVM only moderately while completely inhibiting progesterone-induced GVM and GVBD (Fig. 2A). MPF kinase activation, the definitive parameter of MPF-mediated oocyte maturation, can be measured in vitro by means of kinase activity of oocyte extracts using histone H1 as a substrate [Langan et al., 1989]. We assayed H1 histone kinase activity in v-*erbA*–injected and progesterone-incubated oocytes in three independent ex-

periments with oocytes obtained from different female *Xenopus*. The results of a representative experiment, using oocytes from the same female, are shown in Figure 2B. Marked v-*erbA*–induced nuclear changes, consistent with the onset of maturation, were present 16 h after microinjection (see Figure 3). Therefore, H1 histone kinase activity was assayed over an 18 h period which included and extended beyond the window of potential MPF activation at the start of maturation. In extracts prepared from v-*erbA*–microinjected oocytes, H1 kinase was not activated over this 18 h period when compared with an extract from *lacZ*-injected controls assayed after 2 h (Fig. 2B). The slightly elevated levels of H1 histone kinase activity in samples assayed 4 h and 14 h after microinjection were interpreted as not significant, as they were not reproduced in repeat assays. Moreover, similar fluctuations in H1 histone kinase activity were also observed in extracts of *lacZ*-injected controls assayed over time (data not shown). Compared with a control extract from oocytes cultured in O-R2, extracts from oocytes incubated in progesterone exhibited high levels of histone H1 kinase activity after 2 h (2 h, GVM−), approximately 6 h prior to the appearance of the white spot (8 h, GVM+) (Fig. 2B). Both in the presence and absence of GVM, strong H1 histone kinase activity was present in extracts from progesterone-incubated oocytes over the entire time interval assayed, except for the extract from oocytes lacking a meiotic spot after a 10 h incubation (10 h, GVM−). In summary, these experiments showed that induction of nuclear migration and nuclear instability in the presence of v-ErbA did not involve the activation of the cAMP-regulated CDC2/MPF pathway.

v-ErbA Initiated Changes in the Nucleus and Cortex Characteristic of Early and Intermediate Meiotic Events

Many studies have relied solely on inspection of whole oocytes and dissection of nuclei to assay for GVM and GVBD, respectively. However, this approach does not allow the distinction of complete maturation from arrested meiosis which is also accompanied by nuclear migration and destabilization [Baltus et al., 1973; Steinert et al., 1974]. To enable us to discriminate between these two outcomes in v-*erbA*–injected oocytes, we performed a detailed analysis of semithin sections from v-*erbA*–injected oocytes exhibiting nuclear instability by differential interference contrast microscopy. As shown in Figure 3A, extensive invaginations of the basal nuclear membrane and lamina were visible in v-*erbA*–expressing oocytes 16 h after microinjection. At 36 h, the invaginations appeared as sealed off lacunae of cytoplasm separated by stretches of nuclear membrane, and a yolk-free area was visible in the cytoplasm below (Fig. 3B). The appearance of v-*erbA*–injected oocytes 36 h after microinjection was highly similar to the appearance of progesterone-treated oocytes after 16 h (Fig. 3D). The dynamic rearrangements of the nuclear membrane in v-*erbA*–injected oocytes eventually resulted in membrane rupture at the apical (animal) pole after 42 h (Fig. 3C), and meiotic spindle formation did not occur. In contrast, in progesterone-induced maturation the rupture of the nucleus always occurs in the vegetal half [Baltus et al., 1973; Bement and Capco, 1990; Hausen and Riebesell, 1991]. Further, in progesterone-treated oocytes meiotic spindle formation was observed after 26 h (data not shown). Apical membrane rupture was not observed in *lacZ*-injected controls, demonstrating that rupture in v-*erbA*–injected oocytes was not due to damage during the process of microinjection (Fig. 3E). Nuclear membrane rupture at the apex has previously been shown to be independent of the site of oocyte microinjection and is associated with arrested meiotic maturation [Baltus et al., 1973; Steinert et al., 1974].

The progressive ultrastructural changes taking place in the nucleus and the cytoplasm during maturation have been well characterized [Bement and Capco, 1990] and can serve as markers for temporal subsets of meiotic transformations. As shown in Figure 4A, the vegetal half of the nucleus in *lacZ*-injected controls was extensively folded, forming narrow cytoplasmic channels and larger invaginations extending into the nucleoplasm. Annulate lamellae (AL) were often seen in the cytoplasm, in close proximity to the nuclear membrane. *LacZ*-injected oocytes were identical in appearance to uninjected oocytes (data not shown). In contrast, in v-*erbA*–injected oocytes, the structure of the nuclear membrane was drastically altered. v-ErbA induced the formation of long stretches of adjoined double membranes and the transformation of AL into cisternae (Fig. 4B–E). Seen in

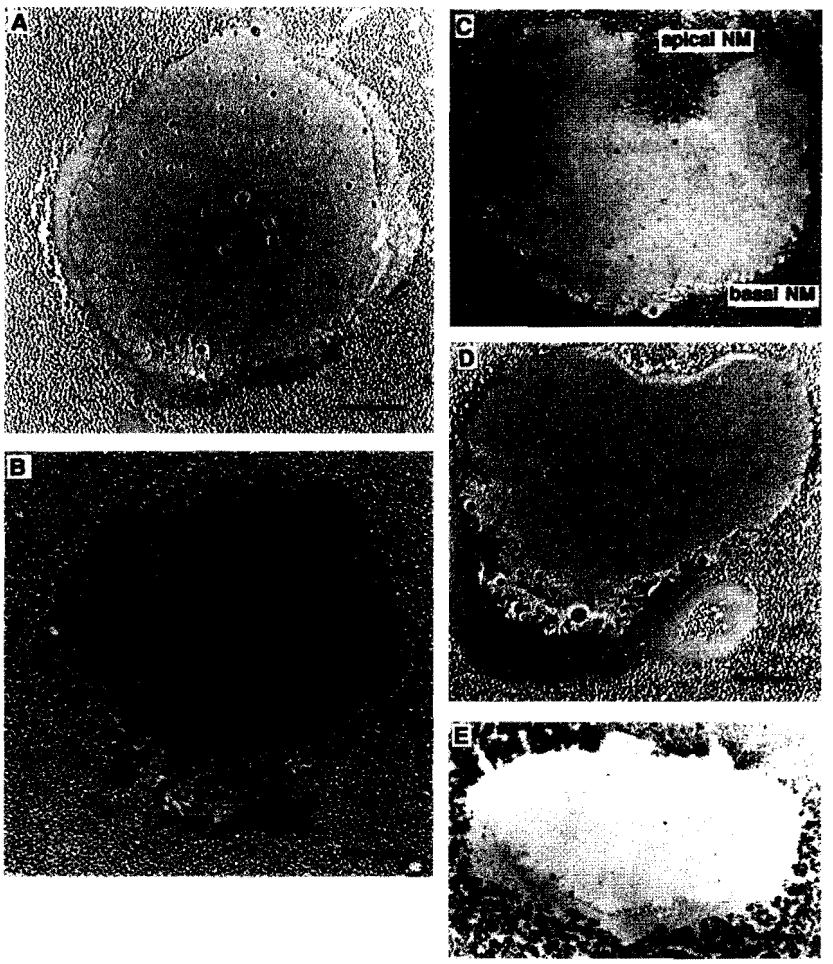

Fig. 3. Semithin sections of nuclei of v-*erbA*–expressing oocytes showed structural changes indicative of meiotic induction, but meiosis was arrested prior to spindle formation. **A:** Invaginations of the basal nuclear membrane and lamina in v-*erbA*–expressing oocytes 16 h after microinjection. **B:** Progressive v-ErbA–mediated structural changes along the circumference of the nuclear membrane and yolk-free zone below the basal nuclear membrane 36 h after microinjection. **C:** Rupture of the apical nuclear membrane (NM) in v-*erbA*–expressing oocytes 42 h after microinjection. **D:** Invaginations of the basal nuclear membrane and lamina and yolk-free space in progesterone-induced oocytes after 16 h. **E:** Absence of nuclear membrane invaginations in *lacZ*-injected control oocytes 36 h after microinjection. Scale bars = 100 μM.

cross-section, the two double membranes appeared tightly adjoined to each other, except for small islands of enclosed cytoplasmic material (Fig. 4D,E). A striking feature of this membrane rearrangement was the alignment of nuclear pore complexes (NPCs) across the joined membranes (Fig. 4F). Furthermore, clustering of NPCs, with adjacent portions of nuclear membrane depleted of NPCs, was seen in the basal regions of the nuclear envelope (Fig. 4G,H). Concomitant with the altered nuclear membrane architecture, multiple layers of cisternae devoid of NPCs developed (Fig. 4I). In addition, the nucleoli showed signs of meiotic transformation (Fig. 4J); that is, the granular and fibrillar parts had become clearly segregated. The rearrangements of the basal nuclear membrane, NPC clustering, AL transformation into cisternae, and segregation of the granular and fibrillar components of nucleoli represent the initiation of events leading to the disassembly of the nuclear membrane and NPCs during meiosis [Bement and Capco, 1990; Brachet et al., 1970; Steinert et al., 1974; Kessel and Subtelny, 1981].

The cortex of oocytes expressing v-*erbA* exhibited a subset of meiotic changes but did not develop the characteristics belonging to the late stage of maturation (Fig. 5A–C). Microvilli, although less numerous and shortened compared to untreated controls (data not shown), were present 36 h after microinjection of v-*erbA* template (Fig. 5A,C), whereas they were lacking

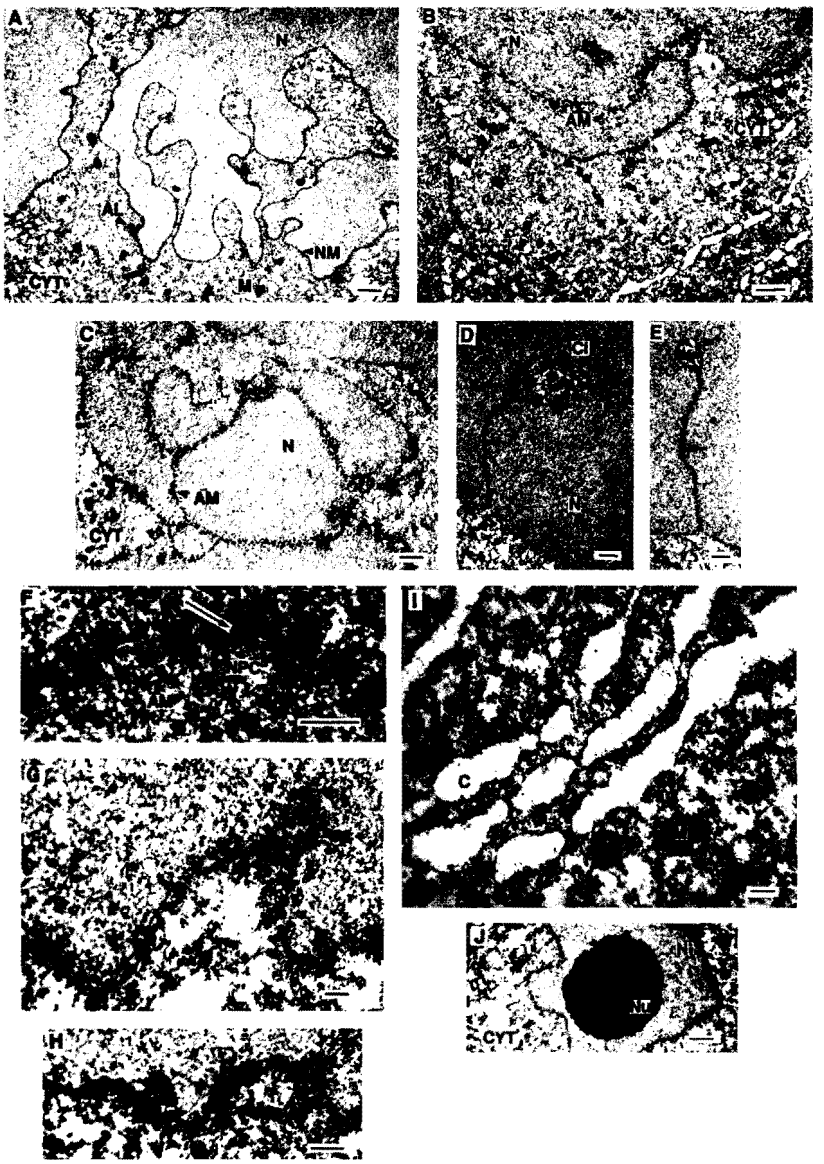

Fig. 4. Ultrastructural meiotic changes in thin sections of nuclei of v-*erbA*–expressing oocytes. **A:** The basal nuclear membrane (NM) of control oocytes injected with 5 ng *lacZ* template was highly folded and formed narrow channels of cytoplasm (CYT) and lobes extending into the nucleus (N). Annulate lamellae (AL) are visible close to the nuclear membrane (en face view). M, mitochondrion. **B–J:** Thin sections of v-*erbA*–expressing oocytes. B,C: Extensive regions of nuclear membrane were tightly adjoined to each other 36 h after microinjection (adjoined membranes, AM). AL had become transformed into cisternae (C). CYT, cytoplasm; N, nucleus. D,E: Long stretches of adjoined membranes (arrows) with aligned NPCs (arrowheads) separated off islands of cytoplasm (Cl) containing mitochondria and other cytoplasmic material. N, nucleus. F: NPCs (arrowheads) were aligned across the adjoined membranes (short arrows) by electron-dense material (long arrow). AM, adjoined membranes. G,H: NPCs in the basal nuclear membrane were clustered (large arrowheads), with adjacent regions being depleted of NPCs (small arrowheads), 36 h after microinjection. I: AL were transformed into cisternae (C) devoid of NPCs. M, mitochondrion. J: The nucleoli (Nu) were segregated into distinct fibrillar and granular regions. CYT, cytoplasm; N, nucleus. A–E,J: Scale bars = 1 μm. H: Scale bar = 500 nm. F,G,I: Scale bar = 200 nm.

after 16 h in oocytes undergoing progesterone-induced maturation (Fig. 5D). Pigment granules were clustered at a distance from the plasma membrane in both v-*erbA*–expressing (Fig. 5B) and progesterone-induced oocytes (Fig. 5E), resulting in formation of a whitish circular spot. In contrast, the majority of cortical granules in v-*erbA*–expressing oocytes remained localized adjacent to the plasma membrane in the area of displaced pigment (Fig. 5B) but were retracted into the interior in progesterone-treated oocytes (Fig. 5E,F). This finding is con-

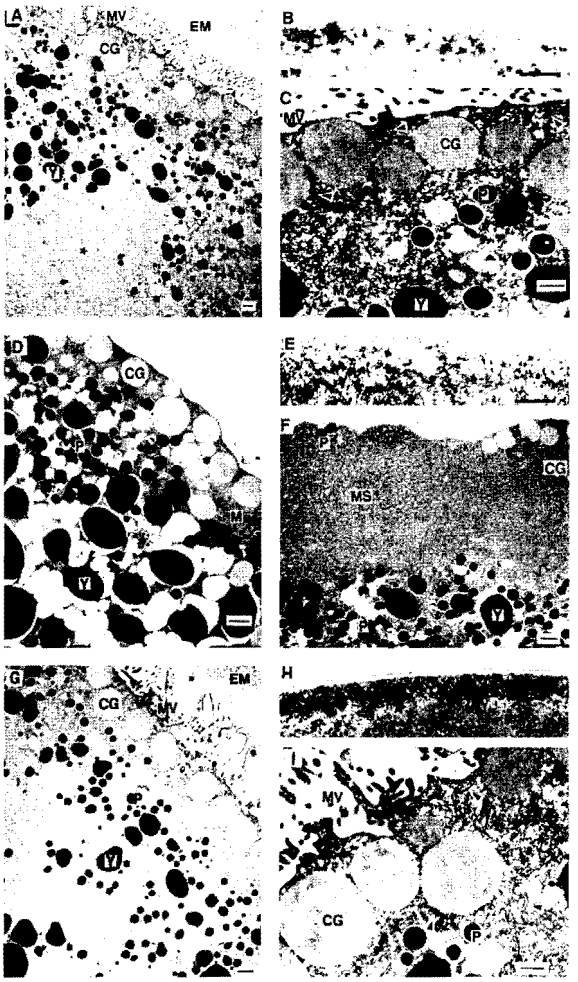

Fig. 5. The cortex of v-*erbA*–expressing oocytes showed a subset of meiotic transformations in thin and semithin sections. **A:** In the cortex of v-*erbA*–expressing oocytes, microvilli (MV) and yolk-free corridors (asterisk) were visible 36 h after microinjection. Cortical granules (CG) were localized immediately below the plasma membrane. EM, extracellular matrix; P, pigment granules; Y, yolk platelet. **B:** Pigment granules were retracted and clustered in the cytoplasm, while cortical granules (arrowhead) were present adjacent to the apical plasma membrane, in the area of the displaced pigment 36 h after microinjection of v-*erbA* (semithin section). **C:** The microvilli of v-*erbA*–expressing oocytes were partially retracted and of low density. The cortical endoplasmic reticulum (arrowheads) had not formed an extensive network and did not encircle the cortical granules. CG, cortical granules; M, mitochondrion; MV, microvilli; P, pigment granules; Y, yolk platelet. **D:** Progesterone-induced maturation resulted in the complete retraction of microvilli after 16 h; yolk-free corridors were disrupted, and mitochondria (M) were randomly dispersed throughout the cortex. CG, cortical granules; P, pigment granules; Y, yolk platelet. **E:** Progesterone induction caused the retraction of cortical granules and the clustering of pigment granules in the cytoplasm (semithin section). **F:** Retraction of almost all cortical granules (CG), pigment granules (P), and other vesicles resulted in an area of clear cytoplasm in the meiotic white spot (MS) of progesterone-induced oocytes after 16 h. Y, yolk platelet. **G:** Cortex of a *lacZ*-injected control oocyte 36 h after microinjection. CG, cortical granules; EM, extracellular matrix; MV, microvilli; P, pigment granules; Y, yolk platelets. **H:** Continuous pigment layer adjacent to the plasma membrane in a *lacZ*-injected oocyte 36 h after microinjection (semithin section). **I:** Numerous extended microvilli (MV) in a *lacZ*-injected oocyte 36 h after microinjection. The cortical endoplasmic reticulum (arrowheads) was comparable to that of v-*erbA*–injected oocytes. CG, cortical granules; P, pigment granules. A: Scale bar = 2 μm. B,E,H: Scale bars = 100 μm. D,F,G: Scale bars = 1 μm. C,I: Scale bars = 500 nm.

sistent with the observation that the rapid decrease in cAMP level triggered by progesterone correlates with the movement of cortical granules away from the plasma membrane, suggesting that cortical granule position is controlled by the level of intracellular cAMP [Bement and Capco, 1990]. Since v-ErbA–induced changes occur without a decrease in cAMP, the cortical

granules would be expected to remain at the plasma membrane. In oocytes microinjected with the *lacZ* plasmid, microvilli were more numerous and further extended (Fig. 5G,I) than in v-*erbA*–injected oocytes (Fig. 5A,C). The layer of pigment granules was continuous in *lacZ*-injected oocytes and located directly adjacent to the plasma membrane (Fig. 5H), differing markedly from the clustered pigment arrangement in v-*erbA*–injected oocytes (Fig. 5B). The structure of the cortical endoplasmic reticulum in *lacZ*-injected controls (Fig. 5I) was comparable to that of the cortical endoplasmic reticulum in v-*erbA*–expressing oocytes (Fig. 5C). Finally, features characteristic of late meiotic maturation—for example, disruption of the yolk-free corridors and development of an extensive cortical endoplasmic reticulum—were not seen in v-*erbA*–expressing oocytes (Fig. 5A,C). A summary of the comparison of ultrastructural changes induced by progesterone and v-ErbA is presented in Table I.

v-ErbA Did Not Act as a Dominant Antagonist of Endogenous TR

Some of the growth-promoting properties of v-ErbA in mammalian cells are mediated by dominant repression of TR and RAR [Sharif and Privalsky, 1991; Desbois et al., 1991a,b; Zhang et al., 1991; Gandrillon et al., 1994]. Stage VI *Xenopus* oocytes contain low levels of TRα and TRβ protein [Eliceiri and Brown, 1994] but lack thyroid hormone [Nagl et al., 1995]. Conceivably, unliganded TR might contribute to the G_2 arrest by repressing certain M-phase–inducing genes. Therefore, we wished to assess whether dominant negative inactivation of unliganded TR by v-ErbA was responsible for the induction of early meiotic events. We have shown elsewhere that the action of endogenous oocyte TR can be blocked by a dominant negative in vitro–generated mutant of human TRβ (TR C122 > A) [Nagl et al., 1995]. When v-ErbA was replaced by the mutant TR C122 > A, the morphology of the basal nuclear membrane in TR C122 > A–injected oocytes was identical to that of untreated G_2-arrested oocytes (Fig. 6). This finding suggests that v-ErbA–induced ultrastructural changes, characteristic of early and intermediate maturation events, were not initiated by v-ErbA acting as a dominant antagonist of endogenous TR.

TABLE I. Induction of Early and Intermediate Meiotic Events by v-ErbA in *Xenopus* Oocytes

Meiotic event[a]	v-ErbA[b]
Early	
AL transformation	+
Basal membrane changes	+
Cortical granule retraction	−
Intermediate	
Nuclear migration	+
Pigment rearrangement	+
NPC repositioning	initiated
Nucleolar dissolution	initiated
Microvilli retraction	initiated
GVBD	apical breakdown
Chromosome condensation	−
Late	
Yolk-free corridor disruption	−
AL absent	−
Cortical ER development	−
Microvilli retraction completed	−
Meiotic spindle	−

[a]Meiotic events were timed [Bement and Capco, 1990] and identified as described [Bement and Capco, 1990; Hausen and Riebesell, 1991; Brachet et al., 1970; Steinert et al., 1974; Kessel and Subtelny, 1981; Kessel, 1992].
[b]The presence (+) or absence (−) of events indicative of different stages of progesterone-induced maturation, in v-*erbA*–expressing oocytes is shown (see Figs. 4, 5).

v-ErbA Was Detectable at Nuclear Pore Complexes

In immunoprecipitation assays, v-ErbA, synthesized in oocytes, is almost exclusively recovered from manually isolated nuclei, while approximately 10% of v-ErbA partitions to the cytoplasmic fraction (data not shown). In oocyte fractionation assays, a protein with a molecular weight of approximately 75 kD was detectable in total soluble protein fractions from isolated nuclei 8 h after microinjection of the gene template (Fig. 7A, panel a). This protein corresponds in size to the full-length *gag*-v-*erbA* fusion protein encoded by the RS-v-*erbA* expression template [Privalsky, 1992]. After 24 h, accumulation of the 75 kD protein in nuclear fractions was further increased, and, surprisingly, the protein was also detectable in total soluble protein fractions from isolated nuclear membranes (Fig. 7A, panel b). To confirm the identity of the 75 kD protein, we carried out immunoprecipitation assays on both fractions using a monoclonal antibody with dual specificity for both v-*erbA* and chicken c-*erbA* [Freake et al.,

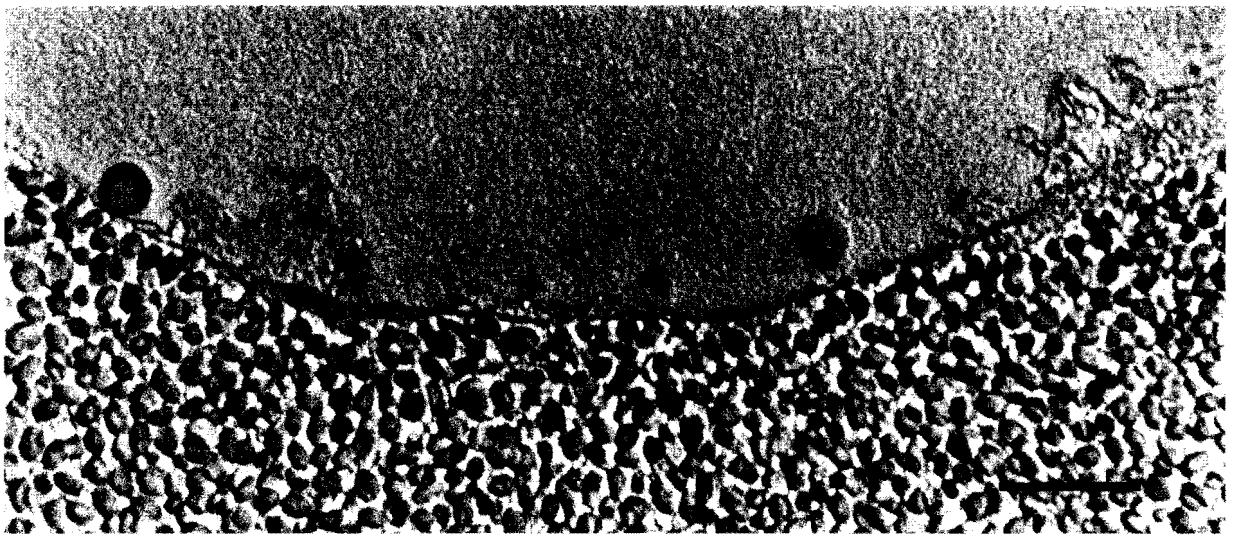

Fig. 6. A dominant negative mutant of TR did not mimic the action of v-ErbA. Semithin section showing the basal nuclear membrane of a TR C122 > A–injected oocyte 36 h after microinjection. Scale bar = 20 μm.

1988]. As shown in Figure 7A, panel c, the 75 kD protein was immunoprecipitated from the nuclear membrane and nuclear fractions. In contrast, deliberate overexposure of the fluorograph showed that wild-type chicken c-ErbA, expressed from injected c-*erbA* templates, was exclusively immunoprecipitated from the nuclear fraction (Fig. 7A, panel d).

To identify the precise location of v-ErbA, we performed immunogold labeling of manually isolated nuclear membranes (Fig. 7B). Nuclear membranes, isolated from v-*erbA*–expressing oocytes, were exclusively decorated with gold particles at the cytoplasmic face of NPCs (Fig. 7B, panels a–c), and, more specifically, the v-*erbA* product was localized to the fibrils emanating from the cytoplasmic annuli (Fig. 7B, panels d,e). A possible reason for the relatively low labeling density, which contrasted with the strong signal obtained by immunoprecipitation, is that the primary antibody was raised against a synthetic peptide of v-*erbA* (residues 58–75) within the DNA binding domain. Since the DNA binding domain is separated from the v/c-*erbA* nuclear localization sequence (NLS) by only 12 amino acid residues [LaCasse and Lefebvre, 1995], antibody accessibility might have been reduced due to steric hindrance caused by interactions between the NLS and a NPC component(s). Control nuclear membranes from uninjected oocytes did not exhibit immunogold labeling with the anti-*erbA* antibody (data not shown), indicating that the observed binding of the gold probes to the cytoplasmic fibrils was specific for the v-ErbA antigen.

DISCUSSION

Our study shows that v-ErbA induces ultrastructural changes in *Xenopus* oocytes characteristic of early and intermediate events of meiosis. This subset of meiotic events was induced by v-ErbA independently of the cAMP-regulated CDC2/MPF pathway and required gene transcription. Importantly, a dominant negative mutant of TR did not induce ultrastructural changes in oocytes, suggesting that this effect of v-ErbA was not mediated by inactivating endogenous TR. This finding implies the existence of a pathway for the partial release of the G_2 arrest in oocytes which can be triggered by the expression of an as yet unidentified v-ErbA–inducible gene(s). The identification of this gene(s) can be expected to provide further insights into the mechanisms through which v-ErbA promotes cell growth.

The rearrangements of the basal nuclear membrane, NPC clustering, annulate lamellae transformation into cisternae, and segregation of the granular and fibrillar components of nucleoli, which were all observed in v-*erbA*–expressing oocytes, represent the initiation of events leading to the disassembly of the nuclear membrane and NPCs during meiosis [Bement and Capco, 1990; Brachet et al., 1970; Steinert et al., 1974; Kessel and Subtelny, 1981]. However, the reorganization of the nuclear mem-

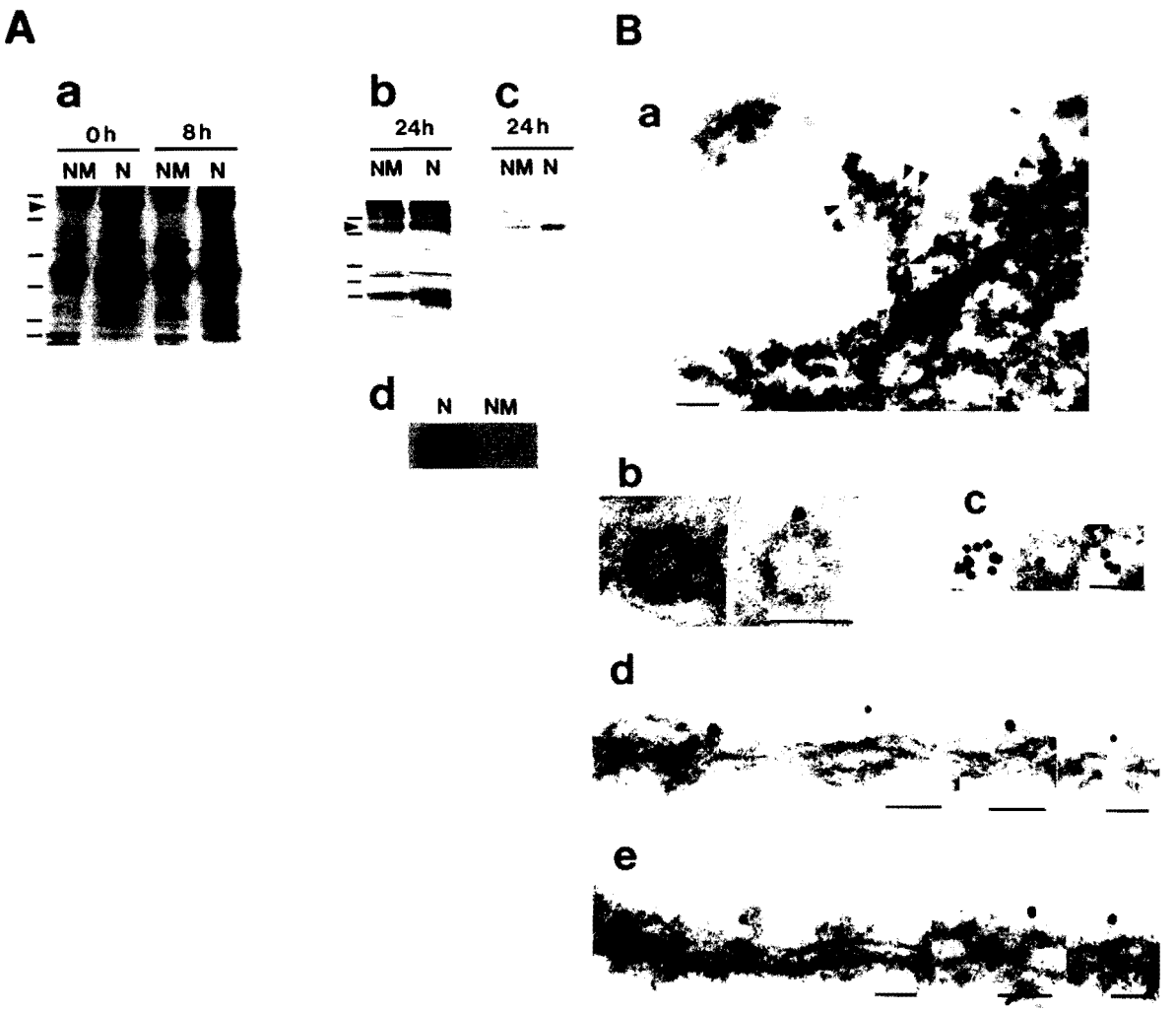

Fig. 7. v-ErbA was detectable at nuclear pores. **A:** Low amounts of v-ErbA were present in nuclear membrane fractions. **Panel a:** Total soluble protein fractions were prepared from ten manually isolated nuclei (N) or nuclear membranes (NM) 0 or 8 h after microinjection of 5 ng v-*erbA* expression template and resolved by 12% SDS-PAGE and silver staining. The arrowhead indicates a protein of approximately 75 kD, corresponding in size to the full-length *gag*-v-*erbA* fusion protein. Molecular weights (D) are indicated by dashes: 85,200, 55,600, 39,200, 26,600, 20,100, and 14,300. **Panel b:** Total soluble protein fractions from ten nuclei or ten nuclear membranes 24 h after microinjection. Symbols are as described in panel a. **Panel c:** The 75 kD *gag*-v-*erbA* protein was immunoprecipitated from nuclear membrane and nuclear fractions using anti-v/c-*erbA* antibodies. Oocytes microinjected with 5 ng v-*erbA* expression vector were cultured in O-R2 medium with 1 mCi/ml L-[^{35}S] methionine for 24 h, and nuclear membranes and nuclei were manually isolated. Nuclear membrane (NM) and nuclear (N) fractions of ten v-*erbA*–injected oocytes were incubated with protein G–Sepharose-antibody complexes in an immunoprecipitation assay. Labeled polypeptides were recovered and separated by 12% SDS-PAGE followed by fluorography. **Panel d:** c-ErbA was exclusively immunoprecipitated from the nuclear fraction. Immunoprecipitation of c-*erbA* protein from the nuclear (N) and nuclear membrane (NM) fractions of 20 oocytes injected with 5 ng c-*erbA* expression vector was carried out as described in panel c. **B:** Immunogold EM detection of v-ErbA at NPCs. Manually isolated nuclear membranes from v-*erbA*–injected oocytes were immunolabeled with 10 nm colloidal gold using a v-*erbA*-specific monoclonal antibody in a biotin-streptavidin bridging technique prior to embedding and thin sectioning. **Panel a:** An en face view of the cytoplasmic side of the nuclear envelope. Arrowheads indicate examples of colloidal gold–labeled NPCs clearly revealed by this plane of sectioning. **Panels b,c:** Selected examples of transversely sectioned NPCs labeled by the anti-v-*erbA* antibody. **Panels d,e:** Representative examples of tangentially sectioned nuclear envelopes. The v-*erbA*–specific antibody exclusively labeled the cytoplasmic fibrils of NPCs. The cytoplasmic side of all cross-sectioned nuclear envelopes faces the top of the figure. The cytoplasmic side of the nuclear envelope often forms blebs (panel e). a: Scale bar = 200 nm. b: Scale bar = 100 nm. c–e: Scale bar = 50 nm.

brane in v-*erbA*–injected oocytes did not proceed past this intermediate stage, and eventually the nuclear membrane ruptured at the apical pole. CDC2/MPF, either directly or through activation of other protein kinases, induces disassembly of the nuclear lamina filaments and NPCs and the vesicularization of the nuclear membrane [reviewed in Smith, 1989; Bement and Capco, 1990], and chromosome condensation and meiotic spindle formation are mediated by the reorganization of microtubules by CDC2/MPF and MAPK [Smith, 1989; Bement and Capco, 1990]. The failure of these progressive events to occur in v-*erbA*–expressing oocytes was correlated with the fact that CDC2/MPF was not activated and that v-ErbA–mediated nuclear changes were not inhibited by taxol. This suggests that the nuclear changes did not involve rearrangements of the microtubular network and, by implication, occurred independently of an MPF/MAPK requirement.

There was a striking match between the v-*erbA*–induced events and oocyte pseudomaturation [Smith, 1989; Baltus et al., 1973; Steinert et al., 1974]. In pseudomaturation, a distinctive subset of structural changes is induced in the absence of MPF activation and de novo synthesis of c-*mos* protein [Drury and Schorderet-Slatkine, 1975; Wasserman and Masui, 1975; Kobayashi et al., 1991]—in particular, adjoined nuclear membranes, mottling of the pigment layer, and condensation of the fibrillar core of the nucleoli [Baltus et al., 1973; Steinert et al., 1974]. Moreover, spindle formation is never observed in pseudomaturation, and eventually apical rupture of the nuclear membrane occurs [Brachet et al., 1970; Steinert et al., 1974; Drury and Schorderet-Slatkine, 1975]. In oocytes, M-phase induction by v-ErbA, like pseudomaturation, is ultimately a destructive process. The reason for this most likely lies in the absence of additional signals from activated protein kinases, essential for the completion of maturation. Once the G_2/M restriction point is passed, the maturing oocyte is committed to undergo events leading to GVBD and lacks the ability to reverse the meiotic pathway. Consequently, the initiated structural transformations eventually lead to the disintegration of the oocyte. This situation is wholly different from the context in which v-ErbA functions in somatic cells. Somatic cells constantly receive a wide range of extracellular signals conveyed by growth factors, which activate mitogenic kinases. Abnormal activation of a gene(s), encoding a putative cell cycle inducer, by v-ErbA can be expected to act in a complementary manner to these events and to enhance certain mitogenic signals. As a result, v-ErbA apparently does not cause nuclear dissolution in somatic cells but confers a reduced requirement for growth factors and an enhanced growth potential [Gandrillon et al., 1987].

In summary, the partial release of G_2 arrest by v-ErbA appears to allow meiotic ultrastructural transformation up to a point where signal transmission by activated MPF and the subsequent phosphorylation cascade become essential for M-phase progression. In somatic cells, v-ErbA cooperates with oncogenic tyrosine kinases, such as v-*erbB*, v-*src*, or v-*sea*, to achieve a fully transformed phenotype [Frykberg et al., 1983; Kahn et al., 1986; Schroeder et al., 1992]. The effects of v-ErbA in initiating the partial release from G_2 arrest in oocytes may be seen as corresponding to the strictly cooperative role of the v-*erbA* oncogene in cellular transformation.

A portion of v-ErbA expressed in oocytes was present at the nuclear membrane at the cytoplasmic face of the NPCs near the cytoplasmic annuli. Specifically, colloidal gold–labeled v-ErbA was detected at the cytoplasmic fibrils of the NPCs, possibly at fibril components, which have been shown to be involved in NLS-mediated ligand docking [Forbes, 1992; Görlich et al., 1994]. That components of the nuclear import machinery may interfere with cell cycle regulation is demonstrated by the effects of a mutation in the *S. cerevisiae* NLS receptor, Srp1, that targets proteins to the NPC [Loeb et al., 1995]. A conditional *srp1* mutant arrests cells at the G_2/M border and was suggested to act by blocking the import of a critical cell cycle regulator. Similarly, Pendulin (or *OHO31*), a *Drosophila* tumor suppressor gene product with sequence homology to Srp1, exhibits cell-cycle-dependent nuclear localization and is required for normal cell proliferation [Küssel and Frasch, 1995; Török et al., 1995]. In addition, other recent studies showed that the fibrils emanating from the cytoplasmic face of NPCs contain the proteins CAN/nup214 [Kraemer et al., 1994] and TPR/nup265 [Byrd et al., 1994], which have both been associated with oncogenesis in humans when fused to other genes. Thus, oncogenes may be capable of inducing oncogenesis by altering the functions of NPCs. Potential effects of v-ErbA at NPCs would be expected to

play an early inductive role, as the association of v-ErbA with NPCs occurs well in advance of meiotic events. While the significance of the localization of v-ErbA at NPCs remains to be determined, it is interesting to consider the possibility that in addition to its intranuclear function in gene activation v-ErbA might modulate nucleocytoplasmic transport of a key cell cycle regulator(s).

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